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Volume XXI OCTOBER 1948 *Number 4*

THE ACTION OF ETHYL CARBAMATE (URETHANE) ON THE RESPIRATION OF ACTIVE AND BLOCKED EMBRYONIC CELLS[1]

JOSEPH HALL BODINE AND LAURENCE ROCKWELL FITZGERALD
Zöological Laboratory, State University of Iowa, Iowa City, Iowa

THE action of narcotics and the part that they play in analyses of respiratory mechanisms of various types of cells have recently been emphasized by Fisher and his co-workers (Fisher and Stern, 1942; Ormsbee and Fisher, 1943; Fisher and Henry, 1943). They have made a detailed study and analysis of the so-called "basal" and "activity" systems of the oxygen-consumption mechanism with a wide variety of materials—yeast, protozoa, sea-urchin eggs, and the like. The results of this work seem of especial interest, not only as a possible aid in further elucidating the process of narcosis, but also in contributing valuable information concerning questions of the relations of various cell activities, especially as they relate to the problems of enzyme activation and energy liberation. These authors point out that narcotics inhibit cell division and other phenomena at concentrations quite different from those seriously affecting the total respiration of the cell. They conclude that the total respiration of these cells is made up of two or more distinct parallel respiratory systems and that the law of mass action does not give a satisfactory description of the observations recorded.

The effects of narcotics and especially of ethyl carbamate (urethane) on the mitotic activity of cells have recently been shown to be of much practical significance in an understanding of various pathological conditions involving cell division (Macht, 1948; Guyer and Claus, 1947). It seems, therefore, highly desirable that further study be made of the effects of urethane on various types of cells and especially on those of an embryonic nature.

The present discussion is concerned with an intensive study of the effects of urethane on the respiratory activity of the isolated embryo of the grasshopper, *Melanoplus differentialis*. This form is of especial interest, inasmuch as isolated living embryos with known temperature and developmental histories are readily obtained (Slifer, 1932). Embryos, free from yolk, are easily taken out of the egg and may be had in relatively large numbers for experimental purposes. Resting or blocked cells, as well as actively divid-

[1] Aided by grant from the National Institute of Health. Acknowledgment is gratefully made to Etta Andrews, John Johnston, and Herman Tharp for technical assistance in carrying out these experiments.

ing ones, are readily available, since no mitotic activity is found during diapause in this form (Slifer, 1931). Blocks to mitotic activity and growth occur during the normal development of the organism, and hence no external agents are necessary in bringing about such conditions. Before the onset of diapause (prediapause) or after the removal of the cell block (postdiapause), actively dividing embryonic cells are always found. Rates of oxygen consumption have been shown to be rather reliable indices of the cellular and physiological states of the embryo throughout its normal course of development (Bodine and Boell, 1934). It was, therefore, thought advisable to test in some detail the action of urethane on the respiratory mechanisms involved in both the blocked and the active states of these embryonic cells, in the hope of throwing further light on the effects of narcotics in general, as well as on the cellular respiratory mechanism in particular.

MATERIALS AND METHODS

Eggs of the grasshopper, *M. differentialis*, were obtained, and dissection of embryos was carried out as previously noted (Bodine and Boell, 1934). Sterile phosphate-buffered Ringer solution, pH 6.8, was used as the suspending medium for the embryos. All solutions of ethyl carbamate (c.p.) were made up in this buffered Ringer solution. Diapause (blocked) and postdiapause (active) embryos were, from all external appearances, morphologically identical (Bodine and Boell, 1936). Only embryos from eggs of known temperature and developmental histories were employed. Diapause eggs were those kept constantly at 25° C. from the time of laying, which were shown by microscopical examination and oxygen-consumption tests to be in the blocked state. Postdiapause eggs were those diapause eggs previously kept at 5° C. for periods long enough to remove the block, which were also checked by further oxygen-consumption tests.

Oxygen determinations were carried out at 25° C. with standard Warburg equipment, using 5-cc. flasks. Control or blank runs on all embryos were for 30 minutes, after which the urethane was added from the side arms, and the oxygen-consumption rates followed for periods of no less than 1 hour. Three banks of manometers, eighteen in number, were used in individual runs, and general averages were calculated. Each concentration was tested many times, so that over-all reading totals for each point represent several hundred determinations. One hundred embryos were used in each flask. In comparing blocked and active embryos, all determinations on a single lot were carried out simultaneously, so that experimental conditions were kept similar throughout.

Concentrations of urethane were calculated from the quantities added to the vessels, and it was found that the degree of effect shown by fifty embryos compared favorably with that for one hundred embryos, indicating that no appreciable amounts of urethane were taken up by the embryos.

RESULTS

EFFECT OF CONCENTRATION OF URETHANE ON THE OXYGEN CONSUMPTION OF BLOCKED AND ACTIVE EMBRYOS

From results of large numbers of experiments involving many embryos, an average curve for the effects of urethane on the oxygen uptake of both blocked and active embryos was constructed and is reproduced in Figure 1. Data from one complete experiment have also been indicated, in order to give some idea of the variations encountered with this material in such investigations.

In general, a rather marked and regular effect of urethane on the oxygen uptake of embryos is noted. Low concentrations up to approximately 0.25 M produce stimulation, while higher concentrations show marked inhibition. These responses are extremely regular and definite. No significant statistical differences between the reactions of the blocked and the active embryos are apparent.

Stimulation by low concentrations of narcotics has been repeatedly demonstrated in a great variety of biological material (Lillie, 1916; Winterstein, 1919). Explanations dealing with the fundamental causes of such a reaction are indeed confusing, if not entirely lacking. For grasshopper embryos, the degree of stimulation for specific concentrations is fairly constant and remains so during the period of exposure to the narcotic. Considerable variation in responses of different lots of embryos occurs, and, as a matter of fact, in some no stimulation at all is apparent. In stimulating or low-concentration ranges of narcotic no inhibition has been noted in either active or blocked embryos.

Inhibition in concentrations greater than 0.25 M occurs in a fairly regular and characteristic fashion, with variations in response much less than for stimulating doses. A slight tendency for active embryos to be more inhibited than blocked ones appears at times, as does the reverse for stimulating doses. No explanation for this tendency is forthcoming at the present.

In all concentrations and exposures employed, practically complete recovery from the narcotic has been found.

RELATION OF OXYGEN UPTAKE, MITOTIC ACTIVITY, AND NARCOTIC CONCENTRATION

In previous reports it has been pointed out that, in blocked or diapause embryos, no mitotic activity can be detected (Slifer, 1931; Bodine and Boell, 1934). Upon removal of the diapause or cellular block, marked mitotic activity at 25° C. is easily demonstrated. The rates of oxygen uptake of blocked and active embryos have been extensively studied, and, in comparable morphological stages, the blocked ones give low and consistent values at 25° C. The active organisms, on the other hand, respire at greatly increased rates, three to four times that for the blocked individuals. These marked differences in respiratory rates seem directly related to mitotic and developmental activities of the embryo.

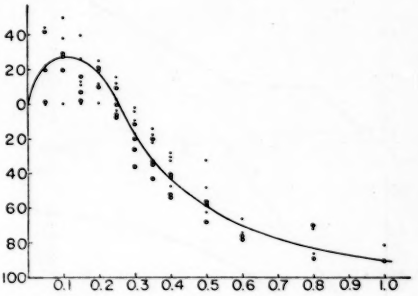

FIG. 1.—Effect of concentration of urethane on the percentage stimulation or inhibition of the oxygen consumption of blocked and active embryos. Ordinate, percentage stimulation (*top*) or inhibition (*bottom*). Abscissa, molar concentration of urethane. Solid curve is average for all experiments. Results for a typical experiment are indicated—solid circles for blocked (diapause), open circles for active (postdiapause) embryos.

Experiments have been carried out on the effects of urethane on the oxygen uptake of blocked and active embryos in comparable morphological stages but with marked differences in oxygen-consumption rates. Typical results of such an experiment are indicated graphically in Figure 2. An inspection of this figure will show that, even though the rates of oxygen uptake of the active embryos steadily increase while those for the

blocked individuals remain constant, the effects of urethane on both are relatively similar. The percentage reductions in oxygen uptake over a period of several days are similar, even though the actual amounts of oxygen taken up in the two cases are quite different.

DISCUSSION

Many theories concerning the action of narcotics on cells have been advanced,

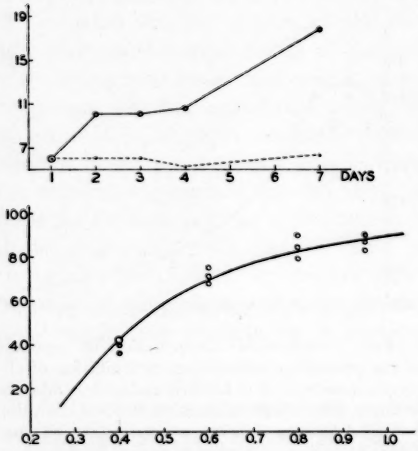

FIG. 2.—Rate of oxygen consumption of blocked and active embryos over period of 7 days and also degree of inhibition of the oxygen consumption by urethane during this period. *Top*, oxygen consumption of embryos at 25° C. Ordinate, O_2 mm³/100 embryos/hour. Abscissa, time in days. Solid circles, blocked embryos. Open circles, active embryos. *Bottom*, inhibition of oxygen consumption at different concentrations of urethane for 7-day period. Ordinate, percentage inhibition. Abscissa, molar concentration urethane. Solid line, average results for all experiments. Points indicate average results for both blocked and active embryo during 7-day period.

and in some cases considerable substantiating evidence has been presented (Lillie, 1916; Winterstein, 1919). No one theory or explanation seems thus far adequate to explain all available data. Among the more recent ideas, those of Fisher and his co-workers (cited above) seem of especial interest, since their approach is from a cellular and quantitative point of view. Briefly stated, Fisher's attention has been directed toward showing that narcotics act on many types of cells and produce a discontinuity in the relation between concentration and effect and that this, in turn, is inconsistent with the view that the inhibition involves only a single respiratory system. His analysis has been confined almost entirely to the inhibiting effects of narcotics, with no attempt at an analysis of their stimulating action. Warburg's (1927) concepts of the action of narcotics on respiration have been expressed by Fisher in the following equations derived from the mass law:

$$E + aUr \rightleftarrows E(Ur)_a \qquad (1)$$

$$\frac{[E]\,[Ur]^a}{[E(Ur)_a]} = K\,, \qquad (2)$$

$$\frac{U}{I}[Ur]^a = K_w\,, \qquad (3)$$

where E = enzyme or catalyst, Ur = urethane, U = uninhibited respiration, I = inhibited respiration, and [ ] = concentration. Data from the present paper have been carefully analyzed according to Fisher's point of view, and what follows is largely concerned with such an analysis.

By plotting the logarithm of U/I against the logarithm of $[Ur]$, a straight line should be obtained if such an equation fits the data at hand. If a single straight line is not obtained, Fisher then concludes that the narcotic inhibits not one system but, instead, two or more parallel respiratory systems, which are independently affected by the narcotic. An inspection of Figure 3—a log-log plot of all data—would lead one to infer that for the grasshopper embryo the mass law seems to afford a satisfactory description of the observations. One might then in-

fer, according to Fisher, that the narcotic (urethane) probably acts upon a single catalyst. Separate plots for blocked and active embryos show no significant deviations from that for the combined data.

Previous results (Bodine, 1934; Bodine and Boell, 1934; Robbie, Boell, and Bodine, 1938) for the effect of cyanide on the oxygen uptake of the grasshopper embryo indicate the presence of cyanide-sensitive and cyanide-insensitive respiratory fractions. During periods of mitotic activity the cyanide-sensitive fraction seems much in evidence, while during the blocked or diapause period the cyanide-insensitive fraction seems to function. Since urethanes are thought to act largely upon dehydrogenases, there is no fundamental reason why results for it on the oxygen mechanism should in any way be similar to those for cyanide.

The marked differences in results for the grasshopper embryo and the data of Fisher for other biological materials are indeed striking. However, differences in the fundamental nature of the materials themselves may well be important factors in any attempt at analysis and comparison. That the effects of urethane on the grasshopper embryo can be completely accounted for on the basis of the existence of only one site of narcotic action seems clearly indicated from the above results. The presence of the block mechanism inherent in the cells themselves may well account for some observed differences between the effects of narcotics on them as compared with cells lacking such a mechanism. It seems reasonable to assume that, in the case of the blocked and active embryos at comparable morphological stages of development, the amounts of enzyme or catalyst involved in respiratory mechanisms would be similar. If such an assumption is correct, then some mechanism involved in the availability of substrate or carrier might well be the important factor. Removing the block or diapause condition could then be interpreted as making additional substrate available and hence an increased oxygen uptake for the active cell. Exactly what the limiting factors are in the process of block is at present so poorly understood that only future investigations can throw further light on these questions.

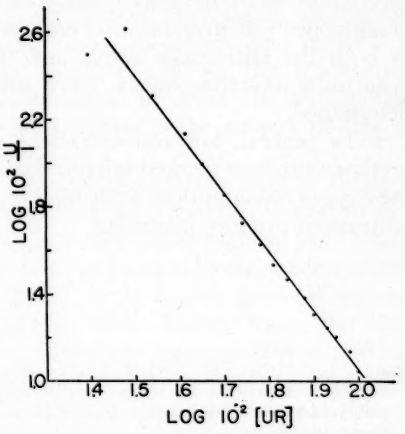

FIG. 3.—A log-log plot, showing relation between ratio of uninhibited (U) and inhibited (I) respiration and concentration of urethane (Ur). Ordinate, log 10^2 U/I. Abscissa, log 10^2 [Ur]. Data represent average inhibitions obtained for all active and blocked embryos as shown in solid curve of Fig. 1.

It has been clearly established that urethane inhibits cell division in most biological forms examined (Taylor, 1936; Fisher and Henry, 1943). In the blocked or diapause grasshopper embryo no mitotic activity is apparent, but the inhibitory effects of urethane on its oxygen uptake are relatively the same as for similar embryos which show marked mitotic activity. If cell division in the grasshopper embryo were considered a process quite separate and unrelated to respiration, one might then well conceive of a rather intricate and complicated

mode of action of the block mechanism, especially as regards the exact site of its operation. For the present and in view of the data presented, however, it would seem that a single respiration system, as indicated for the results above, must be sufficient.

SUMMARY

1. The effects of a series of concentrations of the narcotic, ethyl carbamate (urethane), on the oxygen consumption of grasshopper (*M. differentialis*) embryos, in both the mitotically active and the mitotically inactive stages, have been determined.

2. In general, low concentrations of urethane produce marked stimulation of the oxygen consumption, while high concentrations produce inhibition.

3. No significant statistical differences in response to the narcotic by active or blocked embryos are found.

4. Initial rates of oxygen consumption by the embryos do not seem to be determining factors in the response to the narcotic.

5. Inhibitory effects of the narcotic on the oxygen consumption can be adequately expressed by the equation derived from the law of mass action.

6. No evidence is available for assuming the existence in this form of parallel respiratory systems.

7. Stimulatory effects of low concentrations of narcotic on the oxygen consumption for both blocked and active embryos are definite and regular. No explanation for such reactions is at hand.

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THE EFFECTS OF THYROXINE AND ANTITHYROID COMPOUNDS ON THE SYNTHESIS OF PIGMENT GRANULES IN CHICK MELANOBLASTS CULTURED *IN VITRO*[1]

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The Johns Hopkins University

MANY different kinds of cells have been used in studies on cellular differentiation, but among the most useful are the pigment-producing cells of the domestic fowl. These melanoblasts produce variously shaped and colored melanin granules in accord with their genetic constitution and in response to appropriate factors in their cellular environment (Willier and Rawles, 1940, 1944; Willier, 1942, 1948). Among the factors already proved to be involved in the differentiation of chick melanoblasts *in vivo* are the thyroid hormone and in some breeds the sex hormones, although the mechanism of action of these hormones remains unknown. Willier (1942, 1948) has pointed out that, in addition to hormones and the genotypic constitution of the melanoblasts, the epidermal and dermal components of feather germs are also involved in the production of a specific pigmentation pattern observed in the feather. Recent work with antithyroid compounds (Dieke, 1947; Juhn, 1946, 1947; Lynn, 1948) has further shown that the normal processes of pigment synthesis in melanoblasts can be prevented or changed by several of these compounds. The relationship of these several factors (thyroxine, antithyroid compounds, epidermis, and dermis) to one another and to the biochemical mechanisms involved in melanoblast differentiation is the subject of this investigation.

Abundant evidence already exists to show that the capacity of the melanoblasts to synthesize pigment is to a large degree conditioned by the epidermal substratum of the feather germ (Willier and Rawles, 1940; Wang, 1943; Hamilton, 1941; Watterson, 1942). The importance of the epidermis is further indicated by the observation (Foulks, 1943) that, although reserves of melanoblasts exist in the dermis, the formation of pigment usually begins only after the melanoblasts have migrated into the epidermal components of the feather germ. This epidermal control of melanoblast differentiation is particularly conspicuous in those breeds of chickens which have both red and black melanophores. In these breeds the color of the feather pigment depends upon the epidermal substratum of the feather germ. However, the color and the amount of pigment produced by melanoblasts varies not only according to the particular epidermal environment but also according to the concentration of thyroxine present (Lillie and Juhn, 1932). A deficiency of thyroid hormone brought about by partial or complete thyroidectomy has been observed to

[1] I wish to express my appreciation to Dr. B. H. Willier for helpful discussion during the investigation and for useful criticisms in the preparation of the manuscript. I am also indebted to Dr. W. D. McElroy for stimulating suggestions and to Mr. John Spurbeck for the photographs used. I wish to thank the E. I. Dupont de Nemours and Company for the alpha-naphthylthiourea and the Lederle Laboratories for the thiouracils used in this investigation.

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change the pigmentation of regenerating feathers from white to black, from black to white (Parkes and Selye, 1937; Chu, 1940), and from black to orange, red, or brown (Greenwood and Blyth, 1929; Parkes and Selye, 1937; Blivaiss, 1947*a*, *b*), depending upon the genotype of the chicken concerned. On the other hand, a slight excess of thyroid hormone frequently favors an increase in the proportion of black to the red, brown, or yellow pigment in the plumage of chickens normally elaborating both types of pigment (Horning and Torrey, 1923; Domm, 1929; Danforth, 1933); and, in Barred Plymouth Rocks, mild hyperthyroidism results in an increased deposition of black pigment (Schwarz, 1930; Montalenti, 1934). One investigator (Gericke, 1934) has reported that male Australorp fowls (normally all black) produced reddish feathers when fed thyroid. Large doses of thyroid hormone have been generally reported to produce a loss of pigmentation in regenerating feathers of all breeds tested (Zavadovsky, 1925*a*, *b*, 1926; Sainton and Peynet, 1926; Kříženecký and Podhradský, 1927; Martin, 1929; Hutt, 1930; Emmens and Parkes, 1940).

The problem arises, therefore, as to whether thyroxine acts directly on the melanoblast or indirectly by means of an effect on the epidermal substratum of the feather germ. This problem has been further complicated by the observation that antithyroid compounds not only inhibit thyroxine synthesis but also inhibit or change the color of the hair pigment produced in rats (Richter and Clisby, 1941; Dieke, 1947) and the feather pigment in chickens (Juhn, 1944, 1946, 1947; Domm and Blivaiss, 1944, 1946). The thiouracil-induced pigmentary changes in Brown Leghorn feathers are very similar to the pigmentary changes produced by thyroidectomy and have been attributed to the hypothyroidism caused by the antithyroid drug (Juhn, 1944, 1946, 1947; Domm and Blivaiss, 1944, 1946, 1948).

The principal pigment change observed in rats fed certain antithyroid compounds is a graying of the hair. On cessation of drug feeding, the gray hair is usually replaced by brownish or reddish hair before the normal black is again attained (Richter and Clisby, 1941). Whether these many and varied effects of the thyroid hormone and of antithyroid compounds on pigmentation are exerted directly on the process of melanogenesis or whether the effects are mediated by metabolic processes outside the melanophores can scarcely be answered by such experiments on whole organisms. A more direct approach is required to elucidate the mechanism by which the thyroid hormone and the antithyroid compounds affect the differentiation of pigment. The cultivation *in vitro* of chick melanoblasts provides appropriate material for testing the direct effects on melanoblast differentiation of both the antithyroid compounds and the thyroxine, as well as the effects of dermis, epidermis, and various nutritive components of the culture medium.

MATERIALS AND METHODS

Cells cultured *in vitro* have often served as biological test material. Hamilton (1940, 1941) was the first to test the response of embryonic chick melanoblasts to such synthetic hormones as estradiol, estrone, testosterone, and desoxycorticosterone and also to their solvents—sesame oil and olive oil. In general, the technique described by Hamilton has been used in this investigation.

Since the melanoblasts are very numerous in the back skin of a 6- or 7-day incubated chick, this skin was peeled off

with fine forceps after the edges had been cut free of the adjacent skin with a fine steel needle, sharpened to a knife edge. This rectangular piece of back skin, measuring approximately 10 × 2 mm., was divided bilaterally, and each half was then divided transversely into eight pieces. Thus sixteen pieces of approximately equal size were obtained. Each piece was explanted in a standard hanging-drop culture, composed of one drop each of chicken heparin-plasma, embryonic extract, and Pannett-Compton saline buffered at pH 7.4.

The blood for making plasma was drawn in 30-ml. quantities directly from the hearts of adult Rhode Island Red capons into a syringe containing 5 ml. of a heparin-saline solution. The formed elements of the blood were then centrifuged out and the plasma drawn off and stored in paraffin-lined test tubes in the refrigerator. The embryonic extract was made by first suspending 7-day chick embryos in an equal volume of saline inside of a syringe. The plunger was then used to force the embryos through the fine wire screen which had been placed at the bottom of the syringe. By centrifugation of the resulting tissue brei, the embryonic extract was separated from the residue as a clear supernatant.

The thyroxine, antithyroid compounds, and other chemicals used in this investigation were added to the culture medium in the desired concentration from solutions of the compounds in Pannett-Compton saline. With increasing concentrations of added chemicals, a concentration was usually reached which greatly limited the growth of the explant and changed the normal appearance of the cells. However, all the effects reported in this paper on melanoblast differentiation are restricted to those concentrations of chemicals which did not significantly change the appearance of the culture, except in so far as pigment formation was concerned. All molar concentrations listed are those existing in the culture itself.

EXPERIMENTAL RESULTS

MELANOBLAST DIFFERENTIATION *in vitro*

In order to analyze the effects on melanoblast differentiation of various chemicals added to cultures of chick skin, it was first necessary to ascertain the effect of each tissue component of the skin and of each separable constituent of the culture medium on the synthesis of pigment granules by the melanoblasts. These constituents may be grouped into two major categories: (1) the reacting cells or melanoblasts contained in the explanted skin and (2) the various components of the environment of the melanoblasts—that is, the other cellular constituents of the explanted skin (dermis and epidermis) and the culture medium, made up of plasma, saline, and embryonic extract.

The explanted pieces of skin were very similar in size, degree of development, and genotypic constitution, since pieces of the same size were cut only from the back skin of 7-day Barred Rock chick embryos. Although slight genotypic variations may be assumed to exist in different Barred Rock chickens and although the tissues taken from different regions of the back may not have reached identical degrees of differentiation at the time of explantation, nevertheless, any such differences were too small to interfere with the observations reported in this paper. The uniformity of the control cultures was reflected in the uniformity with which the melanoblasts produced pigment granules. Out of two hundred and eighteen control cultures, only three explants failed to produce any differentiated melanophores within 48 hours from the time of explantation. In a majority

of the control cultures a very few melanoblasts had begun the synthesis of melanin at the time of explantation, and by 48 hours numerous melanophores were visible in nearly all cultures. Pigment synthesis, as indicated by the number and darkness of the granules, normally continued *in vitro* up to about 72 hours, after which time little additional pigment was formed. In order to rule out individual variations when comparisons were to be made of the quantity of pigment formed in two different cultures, the explants in such cases were always taken from a transverse strip of back skin which had been bisected in the midline, one side serving as the control and the other as the experimental explant.

In order to investigate the effect of each tissue component of the explant on melanogenesis, a series of cultures was made in which the epidermis and the dermis were separated by means of fine forceps and explanted in separate hanging-drop preparations. Numerous melanophores developed in both cultures, thus ruling out both epidermis and dermis as individually indispensable for melanogenesis. However, it was obvious that the rate of melanogenesis was not the same in both types of cultures. In all sixty-eight explants of epidermis, the melanoblasts attached to the epidermis developed pigment more rapidly than did the melanoblasts in the comparable dermal explants. Melanoblasts in whole-skin cultures developed somewhat more rapidly than those in dermal explants, but not nearly so rapidly as those in epidermal explants. Although precise measurements of the relative rates of melanogenesis were not feasible in these cultures, nevertheless, it was readily apparent that the melanoblasts in epidermal cultures differentiated into melanophores from 24 to 48 hours sooner than did the melanoblasts in dermal or whole-skin cultures. The presence of dermis next to the melanoblasts in the cultures, therefore, seemed to retard the rate of melanogenesis. Actual physical contact between the dermis and the melanoblasts is requisite for the exertion of this retarding influence, inasmuch as the melanoblasts contained in pieces of epidermis and dermis showed the same differences in relative rates of development, even though the epidermis and the dermis in sixteen cases were placed within 1 mm. of each other in the same culture medium. Thus no readily diffusible inhibitor emanates from the dermis, nor does the epidermis appear to exert any stimulatory influence on melanogenesis. If this were not so, the rate of melanogenesis in melanoblasts in skin cultures would be similar to the rate in epidermal cultures rather than much slower, as is the actual case (see Pl. I, Figs. 4, 5, and 7).

Frequently, in epidermal explants, groups of partially differentiated melanoblasts migrate away from the epidermis and continue to synthesize pigment without direct contact with any other type of cell (Pl. I, Fig. 6). These melanoblasts were easily isolated from 24-hour cultures of epidermis by dissolving away the plasma clot with trypsin and pipetting off the groups of melanoblasts under a dissecting microscope. When these partially differentiated melanoblasts were reimplanted in a plasma-extract–saline clot, additional pigment was synthesized. This demonstrates that the process of melanogenesis can continue in melanoblasts in the immediate absence of all other living systems, though, of course, products previously elaborated by living systems were present in both the plasma and the embryonic extract.

The role of plasma and embryonic extract in the differentiation of melanoblasts was likewise tested by explanting pieces of skin to a medium composed

either of plasma alone or of embryonic extract alone. Out of sixteen explants in plasma, fifteen developed melanophores. However, it was readily observed that the rate of melanogenesis in the plasma cultures was much faster than in either embryonic extract or in the control cultures containing both embryonic extract and plasma. The width of the zone of outgrowth, however, was greater in control cultures than in those cultures containing plasma alone. Although there was little outgrowth from the skin explanted in embryonic extract, this growth behavior is probably attributable to the lack of a solid substrate, since liquid media generally restrict outgrowth in tissue cultures. These observations indicate an "antagonism" between the processes involved in the growth of the explant and those involved in the differentiation of melanoblasts. Such antagonism between growth and differentiation has been previously noted by Doljanski (1930) in the melanin-forming cells of the chick retina.

Although continued growth of cells in tissue cultures required the presence of unknown growth factors present in embryonic extract, nevertheless, pieces of skin explanted to drops of Pannett-Compton saline remained alive for several days and even showed some outgrowth from the margin of the explant. However, no pigment was produced in any of the twenty-four saline cultures examined. After 72 hours in saline, the pieces of skin were transferred to control cultures of plasma and embryonic extract. In twenty out of twenty-four cultures, melanoblasts synthesized pigment, thus indicating that melanoblast differentiation depends upon metabolites which are not present in sufficient quantities in a small piece of embryonic skin.

Since the amino acids, tyrosine and 3,4-dihydroxyphenylalanine, have long been known to serve as substrates for melanin synthesis in certain plants, it was thought possible that they might also serve as melanin precursors in chick melanoblasts. Therefore, each of these amino acids was added singly in 0.002 M concentration to the saline culture medium of eight skin explants. A very few melanin granules developed in a few cells in the saline cultures containing 3,4-dihydroxyphenylalanine, and no melanin granules developed in the cultures containing tyrosine. Apparently, factors in addition to these immediate precursors of melanin are required by melanoblasts for the biosynthesis of melanin granules.

From these observations on the effects on melanogenesis of the various constituents of a standard hanging-drop culture, it may be concluded that the process of melanoblast differentiation in cultures of embryonic skin can take place independently of other living cells in the culture but is dependent upon certain metabolites present in both blood plasma and embryonic extract. Therefore, in investigating the effects of the chemical alteration of the environment on melanoblasts, it may reasonably be concluded that any differences in pigmentation between control and experimental cultures which may result from the addition of various chemicals to the medium may be attributed to a direct effect of the added chemical either on some biochemical system within the melanoblast or on some substance required by the melanoblast for melanogenesis.

MELANOBLAST DIFFERENTIATION IN THE PRESENCE OF ADDED THYROXINE

Several investigators (Zavadovsky, 1926; Schwarz, 1930; Montalenti, 1934) have shown that Barred Plymouth Rocks and other breeds of chickens respond to small doses of thyroxine by increased melanin formation in regenerating feathers, and to large doses of thyroxine by

complete inhibition of pigmentation. If this pigmentation response is due to a direct effect of thyroxine on the melanoblasts, then tissue cultures of melanoblasts in embryonic skin should show the same kind of response to the appropriate concentration of thyroxine. In order to employ concentrations of thyroxine which were physiologically meaningful, a rough calculation was made of the total amount of thyroxine normally present in a chicken. Blivaiss (1947*a*) has shown that a dose of 0.05 mg. of thyroxine injected on alternate days into a completely thyroidectomized Brown Leghorn rooster was somewhat more than adequate for the development of normal pigmentation in the plumage. Since the average thyroidectomized rooster weighed about 950 gm., it may be assumed that the average concentration of thyroxine necessary for the maintenance of normal plumage in the rooster is somewhat less than 5×10^{-6} per cent. The calculation of this concentration is admittedly very rough and does not take into consideration the undoubted differences in the concentration of thyroxine in different tissues. However, since nothing is known concerning the specific concentration of thyroxine available to melanoblasts in a normal chicken, the average concentration in the whole chicken provides the most suitable starting-point around which to vary experimentally the amount of thyroxine available to chick melanoblasts in tissue culture.

A percentage concentration of 5×10^{-6} thyroxine in a chicken is roughly equivalent to a molar concentration of 7×10^{-8} in a tissue culture. The concentration of thyroxine in the plasma-extract–saline cultures was accordingly varied from this "normal" concentration to over fifteen hundred times "normal." Each of the following concentrations of thyroxine was used in twelve separate cultures: 1.1×10^{-7} M, 11×10^{-7} M, 40×10^{-7} M, 167×10^{-7} M, 220×10^{-7} M, 830×10^{-7} M, 1100×10^{-7} M. All the cultures produced numerous melanophores, the small differences in the amount of pigment synthesized by the melanophores in the experimental cultures being very similar to the variation in pigment formation observed in control cultures. It may be concluded that thyroxine does not directly affect Barred Rock melanoblasts. It therefore achieves its influence on pigmentation in adult chickens through the mediation of some metabolic system extrinsic to the melanoblasts and absent from embryonic skin.

INHIBITION OF MELANOGENESIS BY ANTITHYROID COMPOUNDS

Several antithyroid compounds added to cultures of chick skin completely inhibited melanogenesis (see Pl. I, Figs. 2 and 3), whereas others had no effect on pigment formation. All the compounds tested were used in various concentrations in many cultures. In fact, over two thousand tissue cultures of chick skin were examined under the microscope for effects produced by antithyroid compounds on pigment synthesis by melanoblasts. In general, the results obtained with the concentrations reported here were uniform in nature, although occasionally a small number of exceptions were observed. Of the three major classes of antithyroid drugs—thiourea derivatives, thiocyanates, and aniline derivatives (Astwood, 1943; Astwood *et al.*, 1945)—only the class of thiourea derivatives included pigment inhibitors. Nor were all the thioureas effective pigment inhibitors. Thiourea and allylthiourea in 1.67×10^{-3} M concentration and symmetrical-diethylthiourea in 0.67×10^{-3} M concentration did not prevent melanogenesis. Higher concentrations were toxic. The antithyroid compounds which

were found to be pigment inhibitors are phenylthiourea, alpha-naphthylthiourea, symmetrical-diphenylthiourea, propylthiouracil, and thiouracil (Table 1). Although possessing no antithyroid properties, urea, phenylurea, and uracil, because of their chemical relation to the thioureas, were tested in 3.3×10^{-3} M concentrations but were without effect on pigmentation. Goiterogenic compounds found not to inhibit melanogenesis are potassium thiocyanate and the thyroid activity of thiouracil (Astwood, 1945; Vanderlaan and Bissell, 1946), was found to be about five times as effective as thiouracil in inhibiting pigment formation in chick melanoblasts. The thiouracils were not toxic to the cultures even at concentrations as high as 3.3×10^{-3} M, but neither were they strong pigment inhibitors. In fact, phenylthiourea—one of the most potent inhibitors—was over fifty times as effective as thiouracil (see Table 1). The thiouracils further differed

TABLE 1

COMPARISON OF GOITEROGENIC AND MELANIN-INHIBITING POTENCIES OF FIVE ANTITHYROID COMPOUNDS

Inhibitor	Concentration in Tissue Culture	Duration of Pigment Inhibition (Hours)	Relative Goiterogenic Activity*	Relative Potency in Tyrosine-Tyrosinase Systems
Phenylthiourea	0.000055 M 0.000042 M 0.000033 M	>72 48–72 <48	3	1
Alpha-naphthylthiourea	¼ saturated	>72	3(?)	2
Symmetrical-diphenylthiourea	Saturated	>72	4	.....
Propylthiouracil	0.00067 M 0.0005 M	>72 48–72	1	3
Thiouracil	0.0025 M	>72	2	4

* Values from literature.

following aniline derivatives: para-aminobenzoic acid, sulfanilamide, sulfathiazole, sulfapyridine, and sulfadiazine; all were tested in 3.3×10^{-3} M concentrations except the two latter, which, because of their lower solubility, were tested in 0.42×10^{-3} M concentrations.

There is, to sum up, no general correlation between goiterogenic activity and pigment-inhibiting capacity. However, a limited correlation between antithyroid potency and pigment-inhibiting capacity was exhibited by thiouracil and its derivtive, propylthiouracil. The propyl derivative, which has about ten times the antithyroid activity of thiouracil, was found to be about five times as effective as thiouracil in inhibiting pigment formation in chick melanoblasts. The thiouracils were not toxic to the cultures even at concentrations as high as 3.3×10^{-3} M, but neither were they strong pigment inhibitors. In fact, phenylthiourea—one of the most potent inhibitors—was over fifty times as effective as thiouracil (see Table 1). The thiouracils further differed from the thioureas in that subminimal concentrations of the thiouracils added to melanoblast cultures resulted in the surprising formation of green pigment granules in the melanophores. Similarly, a green color developed in tyrosine-tyrosinase systems *in vitro* when partially inhibited by thiouracil or propylthiouracil. On the other hand, subminimal concentrations of phenylthiourea frequently resulted in the differentiation of granules colored various shades of brown instead of the normal black, but no green-colored granules were ever observed in such cultures. At present, no explana-

tion can be offered for the green color, though perhaps the fact that hallachrome (a normal red intermediate in the formation of melanin from tyrosine) is green at a pH greater than 8 may be significant (Mazza and Stolfi, 1931).

In order to test further the reversible nature of the pigment inhibition produced by antithyroid compounds, tissue cultures which had been inhibited for 72 hours were washed in saline for 5 minutes and transferred to a fresh drop of plasma and embryo extract. Within 24 hours melanin had formed in all cultures except those previously grown in the presence of 3.3×10^{-3} M phenylthiourea; however, these retarded cultures also developed melanin, though a longer latent period—48 hours—was required. From Table 1 it may also be seen that the duration of inhibition with phenylthiourea and propylthiouracil is proportional to the initial concentration of the inhibitor.

PIGMENT INHIBITION IN THE PRESENCE OF THYROXINE

In an effort to elucidate the mechanism involved in pigment inhibition by antithyroid compounds, several different substances were added, along with the inhibitors, to the tissue cultures. Especially important is the relation between thyroxine and the antithyroid drugs, since the antithyroid compounds are known to affect thyroxine formation and since both the goiterogenic compounds and thyroxine also affect pigmentation in adult organisms. A number of investigators (Astwood, 1945; and others) have several times demonstrated the absence of any direct effect by the antithyroid drugs on either thyroxine or its metabolic activity. Therefore, the simple fact of pigment inhibition by antithyroid compounds in a tissue culture in which the thyroxine supply was probably normal is adequate evidence for the capacity of the antithyroid drugs to affect directly the pigment formation in adult organisms without reference to the simultaneous effect of the compound on the thyroid gland. Nevertheless, the relationship of these compounds was tested in tissue culture by adding the same concentrations of thyroxine as those previously used to cultures containing phenylthiourea, thiouracil, and propylthiouracil in the minimal concentrations required to inhibit pigment formation for 72 hours. None of the concentrations of thyroxine added to the inhibited cultures affected the inhibition in any way. Therefore, it must be concluded that antithyroid compounds can directly inhibit pigment formation, regardless of the concentration of thyroxine present.

REVERSAL OF INHIBITION BY IODINE

There is no evidence that iodine plays any direct part in the normal pigmentation process, although it is, of course, an essential constituent of thyroxine. However, high concentrations of iodine (0.33×10^{-3} M or greater) were found to inhibit melanin formation in tissue culture. Crystalline iodine implanted under the skin of chickens has previously been shown to have a slightly inhibitory effect on pigment formation in feathers regenerating in the area of the implant, presumably by simulating thyroid hormone effects (cf. Zawadowsky and Titajev, 1928). However, the results obtained in the tissue-culture experiments indicate that the iodine may have produced the pigment inhibition directly—that is, without reference to the role of iodine in thyroxine formation.

Several investigators (Van Winkle *et al.*, 1946) have shown that in human beings previous iodine therapy delays the response to thiouracil. Likewise, DuBois and Erway (1946) have shown that iodine prevents the inhibitory activity of

alpha-naphthylthiourea in a p-cresol–tyrosinase system. Similarly, in the present investigation when iodine in 0.33×10^{-3} M concentration was added to cultures of melanoblasts inhibited by a one-fourth saturated alpha-naphthylthiourea solution, the inhibition was reversed. Furthermore, a ratio of four molecules of iodine to one of phenylthiourea prevented the inhibiting action of phenylthiourea. On the other hand, the addition of iodide (as 3.3×10^{-3} M potassium iodide) to the cultures containing inhibitors did not prevent or reverse the inhibition.

RELATIONSHIP OF ESSENTIAL METABOLITES TO PIGMENT FORMATION AND INHIBITION

Several amino acids and vitamins have been shown to be necessary for normal melanin pigmentation (Groody and Groody, 1942; Pavcek and Baum, 1941; Frost, Dann, and McIntire, 1946). The possibility was suggested, therefore, that the pigment inhibitors might act by depriving the melanoblasts of some essential metabolite (amino acid or vitamin) normally contained in the embryonic extract or in the plasma. In order to test this hypothesis, a number of metabolites involved in melanin formation were separately added to the plasma-extract–saline medium in excess, both alone and in combination with an inhibitor. When added to cultures in the absence of an inhibitor, cysteine hydrochloride and calcium pantothenate in 3.3×10^{-3} M concentration, as well as cystine in 0.28×10^{-3} M concentration, considerably increased the darkness and the number of melanin granules formed, as compared to the normal controls. However, in the presence of minimal inhibiting concentrations of any of the inhibitors, neither of these amino acids nor calcium pantothenate was able to alter the inhibition.

The amino acid, tyrosine, has long been known (Bertrand, 1896) to serve as the substrate for melanin formation in certain mushrooms. The presence of tyrosinase in higher organisms is, however, still a moot question, though evidence has been obtained for the existence of tyrosinase in growing feather germs (Charles and Rawles, 1940). The similarity in structure between tyrosine and phenylthiourea suggested the possibility that phenylthiourea inhibits melanogenesis by competing with tyrosine for the enzyme tyrosinase. This hypothesis was tested by the addition of tyrosine in various concentrations up to the point of saturation (4×10^{-3} M concentration) to cultures containing minimal concentrations of the inhibitors. No reversal of inhibition occurred with the added tyrosine, thus making very doubtful any hypothesis based upon a competition between tyrosine and inhibitor. Furthermore, even in saturated solution tyrosine had no effect on pigmentation in uninhibited cultures.

A more probable pigment precursor in avian melanoblasts is 3,4-dihydroxyphenylalanine (dopa) which has been shown by Raper (1926) to be the first intermediate in the production of melanine from tyrosine in tyrosine-tyrosinase systems. Though the existence of tyrosinase in the skin of higher organisms has not been proved, the presence of a dopa oxidase in the skin has long been demonstrated. Bloch, as early as 1917, postulated the existence of a dopa oxidase to explain the results of his dopa reaction. He found that pigment-producing cells in sections of mammalian skin, when immersed in a solution of dopa, oxidized the dopa to melanin, presumably because they alone contained the requisite enzyme, dopa oxidase. Recently, Herrmann and Boss (1945) have demonstrated the existence of a dopa oxidase in extracts

from the ciliary body and in isolated pigment granules from beef eyes.

Thus, if avian melanoblasts form melanin from dopa rather than from tyrosine and if the inhibition produced by phenylthiourea is due to a competition with dopa for dopa oxidase, then the addition of dopa should reverse the inhibition produced by phenylthiourea. When dopa was added to the culture medium in concentrations of approximately ten molecules of dopa to one of phenylthiourea, the phenylthiourea was unable to inhibit melanogenesis (see Fig. 1). Likewise, a 0.83×10^{-3} M concentration of dopa reversed the inhibition of one-fourth saturated alpha-naphthylthiourea, saturated symmetrical-diphenylthiourea, 3.3×10^{-3} M thiouracil, and 0.66×10^{-3} M propylthiouracil. Each combination of dopa with an inhibitor was tested in a minimum of twenty cultures for each concentration used. The points plotted in Figure 1 represent the result given by at least three-fourths of the cases tested at that combination of concentrations of inhibitor and dopa. Cultures containing concentrations of inhibitor and dopa which placed them on the border line between inhibition and reversal were naturally more variable, but only those points are plotted which were far enough from the border line to give essentially uniform results.

DISCUSSION

MECHANISM OF PIGMENT INHIBITION BY ANTITHYROID COMPOUNDS

The results of this investigation show that several antithyroid compounds can act directly as pigment inhibitors without the mediation of the thyroid gland. The precise mechanism by which antithyroid compounds inhibit the formation of thyroxine and of pigment has not yet been clearly demonstrated. In general, the retardation of chemical reactions may be accomplished by inactivating enzymes responsible for catalyzing steps in the reaction, by breaking links in a chain reaction, or by reducing the concentration of one or more of the reactants. The reversibility of the pigment inhibition by the addition of dopa or iodine or by washing the culture or by the passage of time makes unlikely any mechanism involving an irreversible denaturation of an enzyme. The fact that a particular ratio of concentrations produces a particular degree of inhibition has been commonly used as proof of a competition between substrate and inhibitor molecules for an enzyme (cf. McElroy, 1947, for discussion of mechanisms of inhibition). The effect of dopa in reversing the inhibition when present in a ratio of ten molecules of dopa to one molecule of the inhibitor (phenylthiourea) over a wide range of inhibitor concentrations indicates that there is a competitive inhibition between dopa and phenylthiourea for the enzyme dopa oxidase.

The possibility that tyrosine may serve as the substrate for the formation of both thyroxine and melanin might suggest a common mechanism of inhibition involving tyrosine itself. However, the concentration of tyrosine present in an inhibited culture had no effect whatever on the inhibition, and, furthermore, the competitive type of inhibition, indicated by the reversal of inhibition by dopa, rules out the possibility that the inhibitor blocks the transformation of tyrosine to dopa. If this were not so, the concentration of dopa required to reverse the inhibition would be independent of the concentration of the inhibitor. However, the observation that the duration of inhibition is directly proportional to the initial concentration of the inhibitor makes necessary an additional assumption, if the competitive inhibition mechanism is to be accepted—namely,

that the relative initial proportions of enzyme, dopa, and inhibitor must undergo a progressive change during the life of the culture.

It seems unlikely that the total quantity of enzyme increases significantly in inhibited cultures as compared with control cultures. However, it is quite possible that dopa may accumulate in the melanoblasts or that the inhibitor may gradually be destroyed by the cells in the culture. Melanogenesis would then begin when an adequate concentration of dopa or a subminimal concentration of inhibitor was reached. The fact that the addition of dopa to inhibited cultures reverses the inhibition lends support to the hypothesis that the change in the relative concentrations of dopa and inhibitor is primarily due to an accumulation of dopa in the melanoblasts. Nevertheless, it is the ratio of the concentrations of

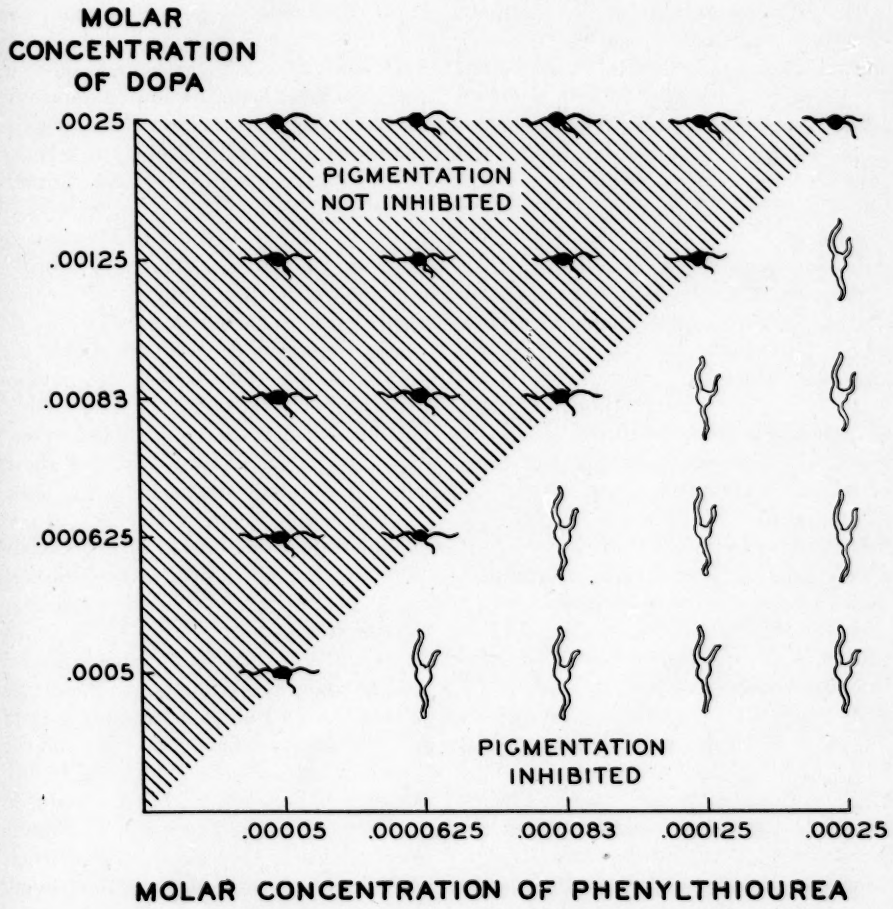

FIG. 1.—Graph showing antagonistic action of phenylthiourea and dopa on melanin formation in chick melanoblasts cultured *in vitro*. Observations on the degree of pigment inhibition were made at 72 hours from the time of explantation. Pieces of back skin from 7-day Barred Rock embryos were used in all cases.

dopa and inhibitor that is significant (see Fig. 1). The observations made during this investigation are consistent with the hypothesis of competitive inhibition. There is, however, a satisfactory alternative hypothesis, based on antioxidant activity, which, in addition, explains how various shades of yellow and brown may be obtained with appropriate concentrations of inhibitors in chick melanoblasts.

In addition, preliminary experiments with the melanin-producing fungus, *Glomerella* sp., have given results which may be explained by the antioxidant hypothesis. When this fungus is grown in the presence of effective concentrations of any one of the inhibitors, the mycelium develops a bright pink color, which gradually changes through various shades of brown to the normal black. This pink pigment is presumably hallachrome—a normal pink or red intermediate in the formation of melanin *in vitro*. It seems probable that the pink coloration of the inhibited fungus mycelium results from an accumulation of hallachrome brought about through a blocking of the polymerization of hallachrome, or one of its derivatives, to melanin.

Competitive inhibition is not the only mechanism of inhibition in which a particular ratio of substrate to inhibitor produces a given degree of inhibition over a wide range of absolute concentrations of the inhibitor and the substrate. A combination of the substrate or one of its derivatives with the inhibitor would also require a particular ratio of substrate to inhibitor to produce any given degree of inhibition. Since the formation of melanin is, in part, an oxidation process, a mechanism of inhibition may be envisaged that involves a combination between intermediate products in the formation of melanin and the inhibitor acting as an antioxidant. Such a mechanism is consistent with all the observations of this investigation, including those of the different pigment colors obtained. The manner of action of antioxidants is not well understood (Mattill, 1947), but it has been postulated (György *et al.*, 1943) that the sulphur-containing antioxidants (thiourea and N-substituted derivatives) are active by virtue of the sulphhydryl residue which is present in the tautomeric form.

Antioxidative activity by N-substituted thioureas would result from the capacity of the sulphhydryl group to reduce activated substrate molecules, in this case intermediate compounds in the formation of melanin, thus breaking the chain reaction. This antioxidant activity of the sulphhydryl group is used commercially in some polymerization processes to terminate chain reactions and thus to limit the average molecular weight of the polymer molecules. Similarly, it is possible that the inhibitors used in this investigation limit the average size of the polymerized aggregations of melanin and thus produce various shades of yellow and brown, depending upon the extent of the polymerization. The antioxidant, when it becomes oxidized, forms a disulphide compound. Werner (1912) demonstrated the formation of this disulphide linkage between two thiourea molecules during the reduction of iodine by thiourea; and recently Miller *et al.* (1945) have shown that iodine will also oxidize thiouracil, joining two molecules of thiouracil through a disulphide linkage. This reaction between iodine and the thioureas explains the reversal of inhibition which is produced by the addition of iodine to inhibited cultures. The iodine simply oxidizes the thioureas until their antioxidant capacity has been destroyed. The fact that several equivalents of iodine were required to reverse the inhibition by the thioureas is in agreement

with the results obtained by Miller *et al.* on the oxidation *in vitro* of thioureas by iodine, in which several molecules of iodine were required to oxidize one molecule of thiouracil.

The formation of melanin from tyrosine is known to involve several oxidation steps (Raper, 1928; Mason, 1948) that might serve as points of attack for antioxidants. Susceptible portions of the chain reaction would appear to be the steps in the oxidative polymerization of hallachrome derivatives to form melanin. The observed dependence of the duration of pigment inhibition upon the initial concentration of the inhibitor and the rapid restoration of the normal rate of melanogenesis, once inhibition has ceased, are readily explained by antioxidant activity of the inhibitors. Bernheim and Bernheim (1942), working with a catechol-(or p-cresol)-tyrosinase-phenylthiourea system, obtained complete inhibition of oxidation for a limited time, after which the oxidation rate abruptly returned to normal. Furthermore, they found that the duration of complete inhibition is inversely proportional to the normal rate of oxidation of the substrate. These results are completely consistent with an interpretation based on antioxidant activity, although the authors proposed an explanation based upon a competitive enzyme inhibition, together with additional assumptions to explain their observations.

In an antioxidant-inhibited system composed of an enzyme and substrate in which the enzyme is not saturated, the addition of more substrate molecules should shorten the period of inhibition by increasing the formation of activated molecules which destroy the antioxidant as they become deactivated by it. Bernheim and Bernheim (1942) found that the addition of substrate shortened the inhibition period in their catechol-tyrosinase systems inhibited by phenylthiourea. Likewise, in the present investigation, the addition of the substrate dopa to inhibited cultures of chick skin containing melanoblasts greatly shortened the period of inhibition.

The inability of tyrosine to reverse the pigment inhibition in tissue cultures may be explained by assuming that tyrosine is not a precursor to melanin formation in avian melanoblasts. However, even though tyrosine were a substrate involved in melanogenesis, it would probably not be a limiting factor, inasmuch as tyrosine is an abundant normal constituent of living matter. Thus the simple addition of tyrosine to the cellular environment would not be likely to produce any effect. Dopa, however, has not been isolated from animal tissue and cannot be considered a normally abundant constituent of protoplasm. The fact that dopa undergoes rapid autoxidation at alkaline pH and the fact that tyrosinase catalyzes a much more rapid oxidation of dopa than of tyrosine suggest the rapidity with which any dopa present in a melanoblast would probably be transformed into melanin.

Inhibition by the thiouracils was somewhat different from that of the other thioureas. The thiouracils were relatively more potent inhibitors of dopa oxidation in the medium, while the other thioureas were far more effective in preventing the formation of melanin granules inside the melanoblasts. These differences may be accounted for by assuming that there are differences among the inhibitors in permeability, activity, and distribution inside the melanoblasts. Phenylthiourea, alpha-naphthylthiourea, and symmetrical-diphenylthiourea must be selectively adsorbed at the surface of the melanin-forming granule because complete inhibition of melanin formation in the melanoblasts is produced by con-

centrations of the inhibitor which do not retard oxidation of dopa added to the medium.

The precise point or points in the chain of oxidation reactions leading to melanin formation at which these inhibitors may act as antioxidants is not revealed by these data, although the preliminary experiments on *Glomerella* indicate that at least one of the points of attack is the prevention of the oxidation of hallachrome. Furthermore, the reddening or browning effect frequently observed in melanophores treated with subminimal concentrations of these inhibitors may be due to an accumulation of hallachrome or to low polymers of hallachrome mixed with black melanin. The fact that complete pigment inhibition may be obtained with higher concentrations of inhibitors shows, however, that one or more points in the chain of reactions before hallachrome must also be affected.

The observation that subminimal concentrations of phenylthiourea lead to the differentiation of brown or yellowish granules instead of the black granules normally found in the Barred Rock melanophores agrees well with the observations of Dieke (1947) and Richter and Clisby (1941) on subminimal effects with the same inhibitor fed to rats. Both papers refer to a brown or brownish-red coloration occasionally replacing the normal black color of the hair as a result of inhibition with phenylthiourea.

Lynn (1948) has recently demonstrated the capacity of several of the antithyroid compounds to inhibit pigment formation in amphibian larvae raised in dilute solutions of the compounds. However, in contrast to the results of the present investigations on chick melanoblasts, Lynn found that allylthiourea, aminobenzoic acid, and sulfanilamide also inhibited pigment formation in amphibian larvae, although phenylthiourea again proved to be the most effective of the pigment inhibitors. The degree of inhibition of pigment production in rats (Dieke, 1947) by phenylthiourea, alpha-naphthylthiourea, and thiourea reveals a close parallel to their relative potencies in inhibiting pigment formation in chick melanoblasts. Similarly, the studies of DuBois and Erway (1946) on the inhibition of oxygen uptake in a p-cresol–tyrosinase mixture by phenylthiourea, alpha-naphthylthiourea, thiouracil, allylthiourea, and thiourea show that these goiterogens have approximately the same relative inhibitory potency in a p-cresol–tyrosinase system as they have in melanoblasts in tissue cultures. In order to determine the relative potencies of these goiterogens to inhibit pigment formation in a tyrosine-tyrosinase system, a series of experiments was carried out in the present investigation in which various concentrations of the inhibitors were added to uniform tyrosine-tyrosinase preparations. The relative pigment-inhibiting capacities in tyrosine-tyrosinase systems (shown in Table 1) were found to parallel closely their relative capacities to inhibit pigment synthesis in melanoblasts. Comparable studies on the melanin-producing fungus *Glomerella*, grown in the presence of various concentrations of the pigment-inhibiting antithyroid compounds, reveal a similar relative activity of the compounds in inhibiting melanin formation in the fungus mycelium—the order of decreasing activity being (1) symmetrical-diphenylthiourea, (2) alpha-naphthylthiourea, (3) phenylthiourea, (4) propylthiouracil, and (5) thiouracil. The striking parallelism of pigment-inhibiting capacity shown by these goiterogens in widely different kinds of melanin-producing systems, as contrasted with the lack of correlation with their goiterogenic activity,

argues for a common specific mechanism of pigment inhibition quite separate from, and perhaps unrelated to, their inhibition of thyroxine formation.

CONTROL OF MELANOBLAST DIFFERENTIATION BY ADJACENT TISSUES AND ESSENTIAL NUTRIENTS

Several investigators (DuShane, 1935; Harrison, 1935; Twitty and Bodenstein, 1939; DeLanney, 1941) have demonstrated the importance of the epidermis in promoting pigment formation in the melanoblasts of amphibians. Epidermal control of the pattern of melanogenesis in the feather germs of chickens has also been shown (Wang, 1943). The experiments reported here indicate that the dermis also exercises a control over the destiny of the melanoblasts by retarding their differentiation. In many breeds of adult chickens the dermis appears to prevent the process of melanogenesis, since (cf. Foulks, 1943) reserves of melanoblasts in the dermis remain undifferentiated unless they migrate into the epidermis. Whether the epidermis of chickens promotes melanoblast differentiation in the same manner as it does in amphibians has not been shown. It seems clear, however, that mature chicken epidermis in some way influences the course of melanoblast differentiation, since the color of the pigment produced in bicolored breeds depends upon the particular epidermal area of the feather germ in which the melanoblast differentiated. The biochemical mechanisms responsible for the antagonism between growth and differentiation may also in part account for the different effects on melanoblast differentiation of the epidermis and the dermis; for, in the adults of most breeds of chickens, differentiated melanophores are to be found largely among the older, more differentiated cells of the epidermis rather than among the dividing cells of the epidermis or the actively metabolizing cells of the dermis.

The fact that chick melanoblasts will not differentiate in a saline culture medium, even though they remain alive, is scarcely surprising, since the melanin pigment is probably made from tyrosine, dopa, or some similar organic compound. However, the capacity to form no more than a very few melanin granules in a saline plus dopa and none in a saline plus tyrosine culture medium indicates that the limiting factors in a saline culture medium include more than the direct metabolic precursors to melanin (dopa or tyrosine). Since analyses by several investigators of the chemical composition of melanin granules (Greenstein, 1948; Serra, 1946) reveal a number of amino acids making up a protein matrix on which the melanin pigment is apparently attached, it is highly probable that the missing factors in saline cultures are those required by the melanoblast for the synthesis of the unpigmented portion of the melanin granule. The few melanin granules observed in saline plus dopa cultures may represent those granules already formed at the time of explantation, which had not, however, produced a coating of melanin pigment. Melanin, of course, can be formed by spontaneous oxidation of dopa in the absence of enzymes. It therefore seems probable that dopa would be readily oxidized to melanin in the melanoblast, presumably on the surface of melanin-producing granules. The controlling mechanism in the final stages of melanin synthesis, with reference to the color and shape of the melanin granules, probably resides in the granule itself.

SUMMARY

1. The object of this investigation was to determine the manner in which thyroxine and antithyroid compounds pro-

duce changes in the color pattern of regenerating feathers in chickens. For this study chick melanoblasts were cultured *in vitro* in the presence of various concentrations of thyroxine and antithyroid compounds.

2. An analysis was first made of the effects of each tissue component of the skin and of each separable constituent of the culture medium on the synthesis of pigment granules by melanoblasts. Unknown metabolites present in both blood plasma and embryonic extract were found to be necessary for pigment synthesis. The presence of other types of cells and tissues was unnecessary for pigment synthesis, and, in fact, the dermis appeared to delay melanogenesis.

3. The general antagonism between growth and differentiation was demonstrated by varying the concentration of embryonic extract added to the culture medium and thus changing the growth-promoting properties of the medium. Melanogenesis proceeded more rapidly in plasma alone, where growth was poor, than in plasma plus embryonic extract, where growth was very extensive.

4. Thyroxine, added to the culture medium, did not influence the synthesis of pigment. Thus melanoblasts in embryonic skin cannot respond to thyroxine, although thyroxine has often been shown to affect pigmentation in adult chickens, probably through the mediation of the adult epidermis.

5. Several members of the thiourea group of antithyroid compounds—phenylthiourea, alpha-naphthylthiourea, symmetrical-diphenylthiourea, propylthiouracil, and thiouracil—directly inhibited pigment formation. The addition of thyroxine to inhibited cultures did not alter the inhibition. Neither goiterogenic aniline derivatives nor thiocyanates inhibited melanogenesis in the tissue cultures.

6. The duration of pigment inhibition produced by the antithyroid compounds was proportional to the initial concentration of the inhibitor. The period of inhibition could be shortened by transferring inhibited melanoblasts to an uninhibited culture medium.

7. The pigment-inhibiting capacity of alpha-naphthylthiourea and phenylthiourea, two of the most potent inhibitors, was destroyed by adding iodine to the medium, presumably through the oxidation of the inhibitor to an inactive compound.

8. Pigment inhibition by antithyroid compounds was reversed by the addition of appropriate concentrations of 3,4-dihydroxyphenylalanine (dopa) to the inhibited cultures. In cultures inhibited by phenylthiourea a ratio of approximately ten molecules of dopa to one of phenylthiourea was required to reverse the inhibition. Tyrosine, cysteine, cystine, and calcium pantothenate had no effect on inhibited cultures, even though they are apparently involved in normal pigmentation.

9. The antithyroid compounds which inhibited pigment formation in chick melanoblasts also inhibited the formation of melanin in tyrosine-tyrosinase systems *in vitro*.

10. The mechanism of pigment inhibition by the antithyroid compounds may be explained either as a competitive enzyme inhibition or, somewhat more satisfactorily, as a direct antioxidative effect on the pigmentation process.

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PLATE I

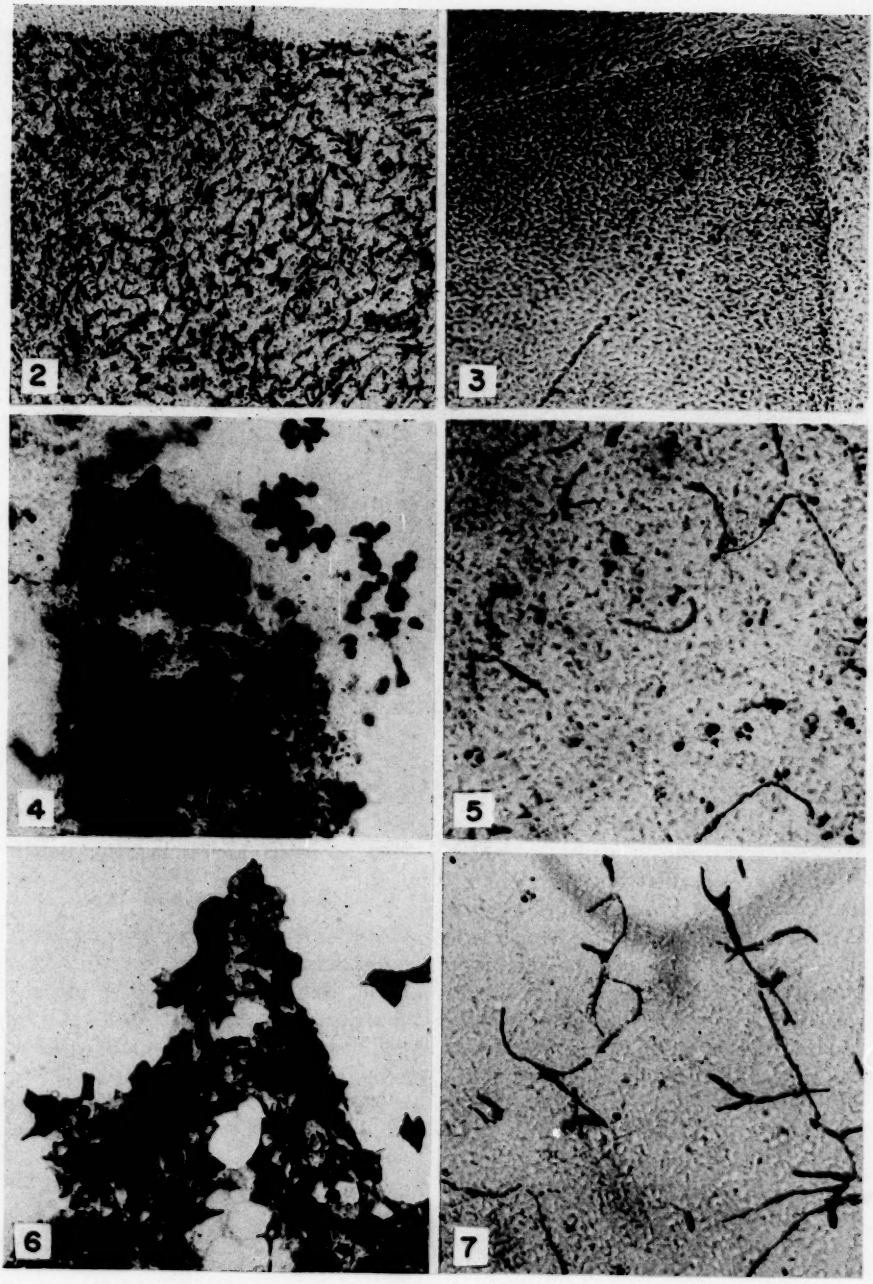

PLATE I

FIG. 2.—Normal development of melanophores in chick skin after 72 hours *in vitro*. Explant made from the unpigmented back skin of a 7-day Barred Rock chick embryo. ×157.

FIG. 3.—Inhibition by phenylthiourea of the formation of melanin in the melanoblasts of 7-day Barred Rock chick embryo skin, cultured 72 hours *in vitro*. Compare with Fig. 2. ×157.

FIG. 4.—Degenerating melanophores in a 72-hour culture of epidermis from a 7-day Barred Rock embryo. Note the great quantity of melanin formed as compared with the melanin in Figs. 5 and 7. ×210.

FIG. 5.—Melanophores in a 72-hour culture of dermis from a 7-day Barred Rock embryo. The dermis was separated at the time of explantation from the epidermis shown in Fig. 4. ×210.

FIG. 6.—A solid mass of melanophores migrating away from an epidermal explant about 48 hours after explantation. Note that the melanophores have not begun to degenerate as they have in the older culture shown in Fig. 4. ×210.

FIG. 7.—Melanophores in an explant of whole skin taken from the same embryo from which the explants shown in Figs. 4 and 5 were obtained. Note that the dermis and whole-skin cultures are approximately the same in melanophore development. ×210.

DIFFERENTIAL OXIDATION AND REDUCTION OF INDICATORS IN RECONSTITUTION OF HYDRA AND A PLANARIAN

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THIS paper is chiefly concerned with the changes in the patterns of intracellular oxidation and reduction of indicators in relation to reconstitution of isolated transverse pieces of hydra and a planarian and their relation to the general gradient pattern of these animals.

MATERIAL AND METHODS

Pelmatohydra, presumably *P. oligactis*, though differing in certain respects from the eastern *P. oligactis* (Child, 1947), from a laboratory culture of the California form, *Chlorohydra viridissima*, collected locally, and a planarian, *Dugesia sp.*, also collected locally, have served as material.[1] The planarian resembles *Dugesia tigrina* in shape of head, but the lateral cephalic lobes are somewhat longer and more pointed, and the animals are more slender and more uniformly pigmented dorsally than is the eastern *D. tigrina*. To the naked eye they appear brown rather than gray. Some data on fission in this form are included in the paper.

The dyes chiefly used as oxidation-reduction indicators are Janus green (diazine green), usually in concentrations of 1/100,000–1/50,000, occasionally somewhat higher; various samples of methylene blue; Sargent "vital stain"; Coleman and Bell "for bacilli"; and other samples of unknown origin, usually in concentrations of from 1/50,000 to 1/20,000. Neutral red, brilliant cresyl blue, and Nile blue sulphate have also been used to some extent for comparison, without essential difference in results.

The indophenol reaction was also used as described in earlier papers (e.g., Child, 1944). Stock solutions consisting of one small drop (approximately 40 drops =

[1] Acknowledgment is due Mr. E. F. Smith, preparator of the School of Biological Sciences, for making most of this material available from cultures maintained by him. The planarian material was obtained from Smith Creek on the western side of Mount Hamilton.

1 ml.) of para-aminodimethylaniline (dimethyl-paraphenylenediamine) in 10 ml. of water and 1 mg. or less of α-naphthol, also in 10 ml. of water, were made daily or oftener. One small drop of each of these reagents in 1 or 2 ml. of water containing the material to be tested, or even lower concentrations, served for the reaction, that is, the intracellular formation of the deep-blue indophenol, catalyzed by an oxidase or by oxidases. The toxicity of the aniline and of intracellular indophenol renders the low concentrations necessary, and even with these concentrations injury and, finally, disintegration occur if the reaction is long continued.

The use of sodium hydrosulphite as a means of obtaining reduced dyes within a few seconds for intracellular oxidation and for reduction of intracellular oxidized dyes has also been described in various earlier papers (Child, 1944, 1947, etc.). A small fraction of a milligram of hydrosulphite removes sufficient oxygen from 1 ml. of dye solution to bring about complete reduction of even the higher dye concentrations and is not appreciably toxic with the exposure periods necessary. With long exposure there is, of course, deprivation of oxygen in cells sealed with exclusion of air. In animals or pieces sealed in 0.5 ml. of completely reduced colorless Janus green; ectodermal oxidation to the red diethyl safranin becomes faintly visible in the more rapidly oxidizing regions in 1–2 minutes, and the color gradually becomes deeper and progresses to other parts, becoming more nearly uniform in 10–20 minutes. Secondary intracellular reduction of the red, partially oxidized dye toward, or even to, colorless may also occur differentially after 10–20 minutes, depending on the amount of hydrosulphite used and the condition of the material. This reduction of Janus green, however, is soon followed by disintegration of the regions concerned. In some cases intracellular oxidation may continue from the red toward the blue-green color of completely oxidized Janus green.

In hydra and the planarian material there is usually no intracellular oxidation in methylene blue reduced by hydrosulphite, except sometimes slight oxidation in the hydra ectoderm. On aeration of the reduced solution to the point at which color just becomes visible, after the material has been in it for a few minutes, intracellular oxidation occurs rapidly with the differential pattern visible for different lengths of time, according to rate and degree of aeration. Methylene blue increases muscular activity and may become intensely irritating, particularly in light. Following intracellular oxidation, more or less reduction may occur in the oxidized solution, apparently in consequence of the irritating effect of the intracellular oxidized dye. Oxidation and reduction can often be repeated several times before reducing capacity is decreased or lost. Brilliant cresyl blue, neutral red, and Nile blue sulphate, oxidized and reduced, have been used less extensively than have Janus green and methylene blue. They give similar patterns but possess no particular advantages, and Nile blue sulphate is more toxic than the others.

Other details concerning use of the dyes for intracellular oxidation and reduction were given in the earlier paper on pattern in hydra (Child, 1947) and in other papers, but certain points require special notice or emphasis. Dyes reduced by hydrosulphite penetrate all parts much more rapidly and uniformly than do oxidized dyes and are much less toxic. After a period in reduced dye with exclusion of air, oxidation shows a gradient pattern, though, if oxygen increase is extremely rapid, as, for example, when the material is brought immediately from the

reduced solution into well-aerated water or is exposed to air in a thin layer of liquid, the gradient may appear only momentarily, with deep uniform, or almost uniform, coloring following almost at once. With the planarian, 15–20 minutes in reduced methylene blue 1/30,000 is sufficient for deep staining of entoderm as well as body wall on oxidation, and with hydra less time suffices. Staining in this way is much more satisfactory as preparation for following reduction than is staining by oxidized dye, which is much less rapid; and with most dyes used there is a marked differential staining, with the result that apical or anterior regions become deeply stained and injured or may even cytolyze, while other parts are still only slightly stained. Use of such material for reduction may lead to incorrect conclusions if not checked by other procedures, because intracellular reduction is retarded or inhibited in the deeply stained regions and the reduction-gradient pattern may be partially or completely the reverse of that in animals not differentially injured. Dye concentrations must, of course, be determined for the particular material.

Janus green in completely oxidized form stains slowly but can be used in preparation for reduction of superficial parts because it apparently stains more uniformly than do the other dyes used, except neutral red, and because the brilliant red color of the partially reduced dye becomes visible after very slight staining. However, the oxidized dye is toxic and must be used in low concentrations.

With very slow and slight oxygen increase, after a period in reduced methylene blue and various other dyes, the oxidation-gradient pattern may be the reverse of that occurring with more rapid and greater oxygen increase. With the very slow increase, oxygen becomes sufficient to permit intracellular oxidation earlier in regions of less rapid, than in those of more rapid, oxygen uptake.

It is also desirable to check patterns obtained by the use of hydrosulphite with reduction patterns appearing with oxygen decrease by oxygen uptake of the material sealed in small volumes of solution. Reduction in these cases is much less rapid than with hydrosulphite but can be hastened by using several pieces or the pieces to be tested together with several individuals. With this procedure, dye concentrations must not be so high that toxic effect occurs before reduction and retards or inhibits reduction differentially or produces cytolysis. In general, satisfactory concentrations of different indicators for different organisms differ widely and can be determined only by repeated experiment.

In estimating color differences in oxidation and reduction, care must be taken to distinguish apparent differences due to differences in thickness of the tissue through which light is transmitted. It is often desirable to compare transmitted light and light largely reflected in a darkened field. Slight color differences are often more clearly distinguishable with widely open diaphragm than they are otherwise.

It seems desirable always to use more than one indicator. Comparison of patterns of oxidation and reduction of dyes with the pattern of indophenol reaction and intracellular reduction of indophenol is useful. Observations on indicator patterns should not be limited to a few individuals or isolated pieces. Many repetitions with variations of procedure are often necessary, and even then questions may remain. It is sometimes difficult or impossible to determine with certainty whether or not apparent slight color differences are actual features of physiological pattern. Also it is often difficult

to determine whether patterns observed in internal organs or tissues are inherent in the parts concerned or are dependent in part or wholly on differential oxygen diffusion from the exterior or on oxygen use by adjoining or surrounding parts. Entodermal patterns are cases in point. In short, it is necessary to learn by repeated experiment with different procedures how to use the indicators with any particular organism.

It is, of course, impracticable to include in the text all the variation in procedure employed with hydra and planarian pieces and in oxidations and reductions of each indicator. However, only those differentials in oxidation and reduction which have appeared consistently in many individual cases with different indicators and various modifications of procedure are considered to be clearly demonstrated features of physiological pattern. These are often visible with a hand lens or even to the naked eye. Cases in which there is uncertainty as to the presence or absence of color difference are regarded as of doubtful significance.

The illustrations represent individual cases observed, but all are similar to numerous other cases. Both shading and arrows are used to indicate the patterns, shading for the regions activated following section and during reconstitution and arrows to indicate direction of progress of oxidation or reduction. The longer arrows indicate more rapid progress, that is, slight gradient differences; shorter arrows slower progress and steeper gradient differences.

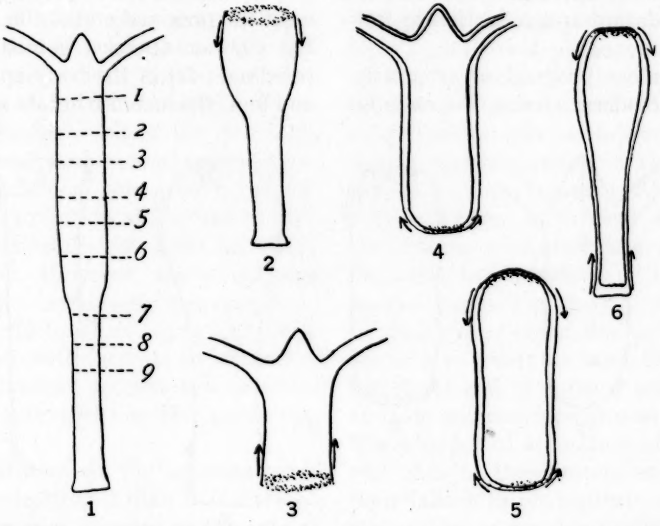
PATTERN IN RECONSTITUTION OF HYDRA

The oxidation-reduction indicator pattern in intact individuals and in developing buds has been described in an earlier paper (Child, 1947). *Pelmatohydra* was the chief material for reconstitution. Interest centers on the earlier stages of reconstitution: with development of hypostome and tentacles, the oxidation-reduction pattern becomes essentially similar to that in later stages of bud development or in intact animals. A total of 52 *Pelmatohydra* individuals were cut transversely into two, three, four, or occasionally, five pieces. Two pieces consisted of *A*, distal to levels *1*, *2*, or *3* in Figure 1, and *BCD*, the rest of the animal. Three pieces consisted of *A*, *B*, or *BC*, between levels *1*, *2*, or *3* and *4*, *5*, or *6* or sometimes *7*, *8*, or *9;* and *CD* or *D* from *4*, *5*, or *6* or from *7*, *8*, or *9* to and including the foot. Four pieces were *A*, *B*, between levels *1*, *2*, or *3* and *4*, *5*, or *6; C* between *4*, *5*, or *6* and *7*, *8*, or *9;* and *D*, proximal to *7*, *8*, or *9*. Five pieces consisted of three entirely in the body, a fourth including the distal part of the stalk, and a fifth the proximal part of the stalk and the foot. However, differences of level, except between body and stalk, were not important factors in indicator patterns. The total number of pieces was 138. A few died or were lost, a few remained for several days without reconstitution, and 125 pieces served for study of oxidation-reduction patterns in earlier stages of reconstitution.

Pieces brought immediately after section into Janus green completely reduced by hydrosulphite in a 0.5-ml. cell, covered and with air excluded, show extremely rapid oxidation to red at the cut surfaces, including both ectoderm and entoderm. The color becomes distinct there in ½–1 minute and earlier than elswhere (Figs. 2 and 3). This is obviously a direct effect of injury and does not differ to a distinct degree at distal and proximal cut surfaces, though it often appears to be slightly more rapid distally than proximally. Less rapidly, a primarily ectodermal gradient of oxidation appears, with the rate of oxidation decreasing from the distal and proximal cut

ends, as indicated by the arrows in Figures 2 and 3. Indicator oxidation is more rapid in these gradients than elsewhere in the pieces, except perhaps in the tentacle tips of *A*-pieces. The gradients represent the spread of the effect of injury and extend only a short distance from the cut surface.

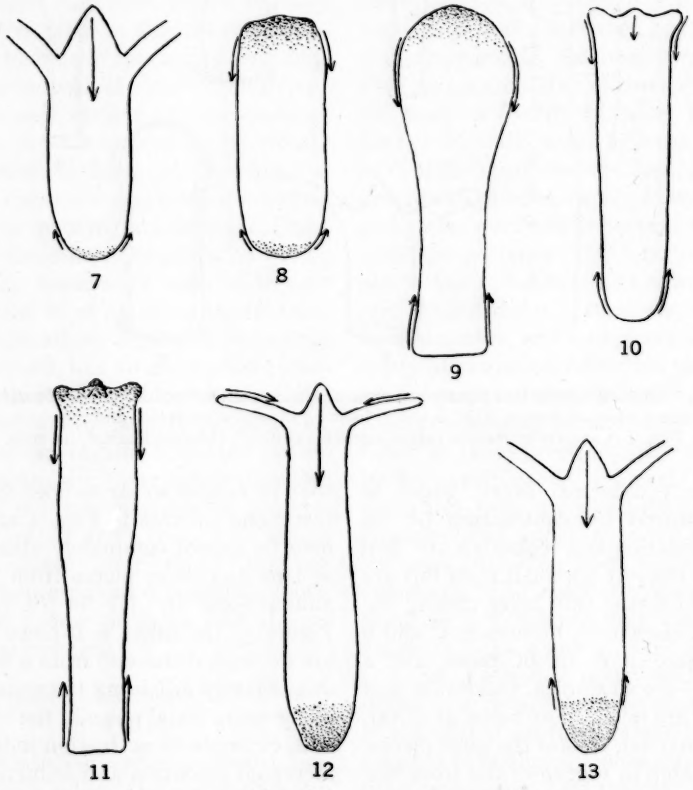
Within 1–3 hours after section, cut ends of pieces are apparently closed by a very thin ectodermal layer, which is often ruptured by contraction of the pieces. Oxidation and reduction are then less rapid than at open cut ends but are more rapid in the thin layer closing the ends than elsewhere. Figures 4, 5, and 6 —an *A*-piece, a *B*- or *BC*-piece, and a *CD*-piece—are examples. Oxidation and reduction are much more rapid at distal, than at proximal, ends of the same pieces, as is indicated in Figure 5; and from distal ends a gradient of decreasing rate follows the primary reaction within a few minutes (Figs. 5 and 6). In reduced Janus green, oxidation in the thin membrane is often followed by reduction to colorless, while other regions remain red. This is less frequent or does not occur at proximal ends and is regarded as indicating more rapid oxygen uptake at the distal end. There is, in general, less evidence of activation at proximal, than at distal, ends of pieces, and the acropetal gradient from a proximal end does not appear so soon or extend so far as does that at the distal end (arrows in Figs. 4 and 5) and may be almost completely absent. Even in two successive pieces from the same animal—one an *AB*- or *BC*-piece like Figure 5, the other a *D*-piece like Figure 6—with distal end from a body-level immediately adjoining the proximal end of the more distal piece in the intact animal, evidence of activation indicated by increased oxidation and reduction is distinctly less at the proximal end of the more distal, than at the distal, end of the piece immediately proximal to it (Figs. 4,



FIGS. 1–6.—Oxidation-reduction patterns in the earlier stages of reconstitution of *Pelmatohydra*. Fig. 1, outline indicating levels of section. Figs. 2 and 3, increase in rate of oxidation and reduction immediately after section. Figs. 4–6, greater increase in rate of oxidation at distal, than at proximal, cut ends.

5, and 6). Evidently it makes a difference, not only in morphogenesis but in oxidation-reduction of indicators, whether a particular level of the original animal constitutes the distal or the proximal end of a piece. This difference is an expression of the gradient pattern in the body of the intact hydra and of the distal-proximal dominance. In *D*- or *E*-pieces, entirely within the stalk, isolation by section usually does not appreciably increase the rate of oxidation and reduction of indicators.

With further progress of reconstitution, the ectoderm closing the ends becomes thicker. At 24 hours after section, increase of oxidation at the proximal ends of *A*-pieces is still slight, and the acropetal gradient extends only a short distance (Fig. 7). Oxidation and reduction are more rapid in the distal region of a *B*- or a *BC*-piece from the same animal, and the gradient of increased rate extends proximally a considerable distance (Fig. 8). At the proximal end of this piece, also, there is only a slight increase, as at the proximal end of the *A*-piece. In the *CD*- or *D*-piece, consisting of the proximal part of the body and the stalk and foot, the increase in rate at the distal



FIGS. 7–13.—Later stages of reconstitution. Fig. 7, very slight activation at proximal cut end. Figs. 8 and 9, much greater activation at distal end with new gradient. Figs. 10 and 11, early tentacle development, with new gradient in each tentacle bud. Figs. 12 and 13, early stages of stalk and foot development.

end is again much greater than at the proximal end of the piece immediately distal to it (Fig. 9). In some cases oxidation has seemed to be slightly less rapid at the distal ends of *CD*- or *D*-pieces than at distal ends of *B*- or *BC*-pieces, which are from a higher-gradient level in the body; but the difference, if real, is slight, as might be expected, since the gradient in the body of the intact animal is slight.

Within 48 hours after section, hypostome and tentacle buds are usually well defined in pieces with distal reconstitution. As in buds, the developing hypostome is the high end of the new body gradient, and each tentacle bud represents an additional new gradient of still more rapid oxidation and reduction, decreasing basipetally from the tip (Figs. 10 and 11). However, the developing tentacles are extremely susceptible to injury by the oxidized dyes and indophenol; and, with increase in intracellular concentration, reduction is retarded or occurs only with, or just preceding, cytolysis.

Reconstitution at the proximal ends of pieces is less rapid than it is distally. There is gradual increase in the rate of oxidation and reduction (Fig. 10, 48 hours); and three days after section there is usually a well-defined new gradient at proximal ends, as reconstitution of stalk and foot progress (Figs. 12 and 13). These patterns were observed with development at room temperature varying little from 20°–22° C.

It is evident from the oxidation-reduction patterns that in the earlier stages of reconstitution a new gradient with higher gradient levels is being superimposed from the distal end on the part of the original gradient present in the piece, that is, the distal region of the piece is becoming more distal physiologically and capable of developing into an apical region. This change precedes any visible morphological reconstitution. More gradually a new acropetal gradient pattern, representing development of stalk and foot, is imposed on the proximal regions of pieces with cut proximal ends. After 3 days, or even earlier in some pieces, the pattern becomes essentially like that of the bud after the stage of appearance of tentacles (Child, 1947) and requires no further consideration here.

A few additional points require mention. Very short *A*-pieces, cut almost immediately proximal to the tentacle bases, show only the increased oxidation and reduction immediately following section. No distinct activation of the proximal region and no reconstitution of more proximal parts have been observed in these pieces, even after other pieces from the same individuals have reached advanced stages. The absence of activation in these pieces is not due to inability of the cells to react; the same body-level at the distal end of a piece undergoes activation and develops into an apical end. The absence of activation at the proximal ends of these pieces suggests that their failure to reconstitute more proximal regions is not due to differentiation but to dominance of the apical region, which prevents the physiological alteration necessary to initiate reconstitution.

Considerable variation in rate of reconstitution was observed. Some of the shorter pieces remained for several days with no evidence of reconstitution and sometimes without closure of the cut ends. Such pieces, even after closure of the ends, showed little or no evidence of increase in rate of oxidation and reduction. Some of them finally showed a gradual increase and began to develop slowly. In others, which remained ovoid or almost spherical, no evidence of gradient pattern could be observed; oxidation and reduction occurred uniformly over the whole ectoderm after 2–3 days.

Such pieces had apparently lost their physiological polarity. Complete or almost complete absence of activation, as indicated by absence of increase in rate of oxidation and reduction at distal cut ends in the stalk region, and absence of reconstitution are apparently correlated; and here also the question arises whether the stalk cells are so highly differentiated that they cannot react or whether they are under the dominance of the more active proximal region and the foot, which is apparently a highly active region (Child, 1947).

A differential secondary reduction in oxidized methylene blue usually occurs. The regions activated following section or later in the process of reconstitution reduce most rapidly, until reduction is retarded or inhibited by increasing intracellular concentration of the dye.

The rate of entodermal oxidation apparently increased adjoining distal cut ends, but no appreciable increase has been observed proximally. Entodermal reduction occurs very slowly in intact animals (Child, 1947); whether it becomes more rapid in reconstitution remains uncertain because of the difficulty in observing color changes in the deeply colored body entoderm.

In the course of observations it was noted that the *A*-pieces seemed to be much more active than did the distal regions of intact individuals. This observation led to the inclusion of intact single individuals as controls in the same cells with single *A*-, *B*-, or *BC*-, and *CD*-pieces at stages 24 and 48 hours after section in Janus green completely reduced by hydrosulphite. In 14 such pieces—8 from four animals cut into 2 pieces each and 6 from two animals cut into 3 pieces each—oxidation of Janus green was distinctly more rapid than in corresponding regions of intact controls. In 4 pieces, 2 each from two animals, stained with oxidized Janus green 24 and 48 hours after section, reduction by hydrosulphite was more rapid than in corresponding regions of similarly stained intact controls. In one *B*-piece from the middle of the body, contracted and without evidence of reconstitution 2 days after section, intracellular reduction of fully oxidized Janus green was less rapid than in a control. These cases—all that were tested with controls—suggest that change in physiological condition in the isolated pieces in good condition is not limited to the regions adjoining the cut end or ends but involves the entire piece to some degree. Even in the *A*-pieces, the intact, fully developed tentacles and hypostome show more rapid oxidation than did these regions in the controls. In these pieces there may be no reconstitution, but the almost continuous motor activity probably determines the difference from controls. In pieces from more proximal levels there seems to be much less motor activity than in intact animals, at least during the earlier stages of reconstitution; here the difference from controls is evidently associated with reconstitution. The piece, undergoing reconstitution into a new individual, like the bud developing into a new individual, oxidizes more rapidly than does the fully developed animal and is to be regarded as physiologically younger.

Incidental observations on oxidation and reduction in testes which appeared on many *Pelmatohydra* individuals of the laboratory stock during the autumn of 1947 are briefly noted. The indicators, even when reduced, penetrate the closely packed cells of the testis interior less rapidly than they do the general ectoderm; consequently, unless sufficient time is given in reduced dye, oxidation is less rapid in the testis interior than in general ectoderm. With longer exposure periods in reduced dye, testis oxidation is dis-

tinctly more rapid than in general ectoderm adjoining, and reduction following oxidation is also more rapid than in the ectoderm. The covering ectoderm of the testis reduces basipetally from the tip, the "nipple" in later stages. According to observations repeated on many testes in early and late stages of development, oxidation and reduction progress basipetally throughout the testis interior in earlier developmental stages, provided that sufficient time for penetration of the dyes is given. In advanced testes, however, acropetal progress of oxidation and reduction has been observed in numerous cases. It is suggested that this difference in earlier and later stages may be associated with the gradation in the testis of stages of spermatozoan development. In earlier stages there is presumably decrease basipetally in growth and developmental activity throughout the testis interior. Fully formed spermatozoa first appear in the distal region of the testis. In later stages the more distal levels are differentiating, or have differentiated, into spermatozoa, while more basal levels may still be growing and dividing.

A few series of isolated pieces of *Chlorohydra* showed indicator patterns similar to those of *Pelmatohydra*, but with more rapid reconstitution.

Perhaps the most important points in these observations on indicator patterns in isolated pieces of hydra are, first, the determination of a new gradient in the distal region of the piece, which makes this region more distal, and more gradually another less distinct gradient in the proximal region, associated with proximal reconstitution; second, the absence of reconstitution when no gradient of increased rate of oxidation and reduction appears. The new gradients appear before there is any evidence of morphological reconstitution and, unless they are without any physiological significance, appear to be initiating factors in the reorganization which follows their appearance and coincides in localization with them.

PLANARIAN PATTERNS

INTACT ANIMALS

Indicator patterns of the intact planarian have been previously determined only in part. Some fifteen years ago it was found that in *D. dorotocephala* reduction of Janus green in the unpigmented ventral body wall decreased in rate from the head to the level of the fission zone and showed a distinct increase posterior to this level, i.e., in the posterior zoöid or zoöids (Child, 1941, pp. 110, 111). The posterior zoöid is not evident morphologically, though, in laboratory stocks which increase in length slowly and become very long without fission, it often becomes independent of more anterior regions in motor reactions, and, in consequence of attachment to the substrate while the anterior zoöid attempts to continue forward locomotion, the body immediately anterior to the posterior zoöid becomes permanently elongated and more slender than the rest of the body. The posterior zoöid is indicated by a higher rate of oxygen uptake and CO_2 production than is the region immediately anterior to it, by a higher susceptibility to toxic effects of a large number of chemical and physical agents, and by higher reconstitutional capacity (Child, 1941, pp. 108–17, 177–90, 321–23, and papers by Child and by Hyman cited there).

In the California planarian serving as material for the present study, the posterior zoöid is apparently less developed than in *D. dorotocephala*. No cases of recent fission have been found in newly collected material, though such cases are very common in *D. dorotocephala* and also in *D. tigrina*. No fissions have oc-

curred in laboratory stocks except in two individuals after several weeks without food. Many years ago it was found that fission could be induced in the longer individuals of *D. dorotocephala* to almost, or often to quite, 100 per cent by removal of the head. With this procedure, fission occurred several days after the head was removed, when the new head had developed far enough so that the animals began to move about again "spontaneously." In the largest animals one removal of the head was usually sufficient, but with small animals two or even more removals might be necessary. Fission could be induced in this way in animals only about half the length at which fission occurs in nature (Child, 1910). In the largest of the California planarians (12–15 mm. in length) no fissions occurred after a single removal of heads; but, after a second removal of the regenerated heads, fissions finally occurred in all ten of the animals comprising the experimental lot, and in all cases fission was at approximately the same body-level as in *D. dorotocephala*. This difference in development of the posterior zoöid may be due to environmental conditions. Perhaps, with increase in length, fission might occur more readily, but thus far laboratory feeding has not led to increase beyond 15–16 mm.

The indicator patterns of the California planarian have been more extensively investigated than in other species, with material consisting of fifty-one intact animals of various sizes and many pieces in all stages of reconstitution. The intracellular indophenol reaction and oxidation of reduced dyes in the presence of sufficient oxygen are most rapid in the tip, margins, and cephalic lobes of the head. The rate of oxidation decreases posteropetally in the body wall. In the posterior zoöid region, oxidation seems to be slightly more rapid in many cases, but the difference, if real, is very slight and is regarded as uncertain. At all levels the margins of the body and the ventral body wall oxidize indicators more rapidly than does the dorsal body wall. A very distinct increase in rate appears at the extreme posterior end, presumably a region of growth.

After very slight indophenol reaction or very slight staining with oxidized Janus green 1/100,000, the head reduces the indicator more rapidly than other regions do, and reduction progresses posteropetally, at least in the anterior zoöid with a slight gradient. It has not been possible, however, to obtain more rapid reduction in the head than in postcephalic regions with any concentration of methylene blue from 1/200,000 up to 1/20,000. With all oxidized concentrations, great irritation is indicated by extreme extension and contraction, absence of co-ordinated motor activity, and turning on the side or with ventral surface up. The head is clearly much more susceptible to toxic effects of methylene blue than other parts are. It very soon becomes contracted and rounded, and the cephalic lobes contract so strongly that they do not protrude beyond the general outline of the head. Under these conditions reduction in the ectoderm and apparently in the muscular layers of the head is greatly retarded as compared with the body. With increasing intracellular concentrations of indophenol, oxidized Janus green, and other dyes, similar retardation of reduction occurs. The head may even begin to disintegrate from the tip posteriorly and from the cephalic lobes while the body is still completely intact and capable of rapid reduction. However, even after reduction is retarded or inhibited in the superficial tissues of the head, it may still occur rapidly in the ganglionic region, as is described below; intracellular concentra-

tion of indicators is doubtless much lower there than at the surface of the head. Irrespective of the results with indicators, metabolism is unquestionably higher in the head region than in the body; but the high toxicity of indicators, particularly methylene blue, as well as the rapid staining of the predominantly sensory tip and margins, suggests a more or less specific sensitivity or perhaps a high permeability to the oxidized dyes associated with the sensory character of these regions.

Posterior to the head, the reduction of indicators before toxic effect appears

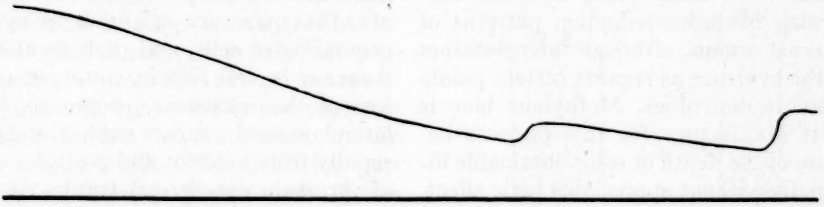


FIG. 14.—Oxidation-reduction gradient of the surface and body wall of the intact planarian; anterior end at the left. Rates of oxidation and reduction decrease from the anterior end, with a slight increase in the posterior zoöid and further increase at the extreme posterior tip.

progresses posteropetally to a level a short distance posterior to the mouth, i.e., the level at which fission has been found to occur. Posterior to this, reduction seems to be slightly more rapid in the longer animals; but in animals 7–8 mm. in length no evidence of more rapid reduction in this region has been obtained. This agrees with other evidence concerning development of the posterior zoöid in relation to length of the animal. The extreme posterior tip reduces almost as early as the head does, until such reduction is retarded or inhibited by toxic effect.

After intracellular reduction and oxidation of methylene blue have been repeated several times, the toxic effect and retardation of reduction extend to increasing distances posterior to the head, and the reduction gradient in the body may become more or less completely reversed in direction. More or less reversal of the oxidation pattern may occur with gradual and slight oxygen increase in reduced methylene blue solution because the oxygen concentration permitting intracellular oxidation is attained sooner in regions of lower, than in those of higher, oxygen uptake. The oxygen concentration permitting oxidation of completely reduced Janus green to the red diethyl safranin is so low that, even with very slight or gradual oxygen increase and usually in completely reduced solution with air excluded, intracellular oxidation takes place.

The results obtained with indicators are believed to justify the conclusion that intracellular oxidation and reduction preceding toxic effects progress posteropetally in the planarian body wall, with apparently a slight increase in rate in the posterior zoöid region in the longer animals and a very distinct increase at the extreme posterior tip, and that, at any body-level, oxidation and reduction are more rapid ventrally than dorsally. The longitudinal gradient pattern is represented schematically in Figure 14, the anterior end being at the left. The curve serves for both oxidation and reduction, greater distances above the base line indicating higher rates. The increase in rate indicated for the posterior zoöid is

regarded as justified because the different lines of evidence already noted and to be noted agree in indicating the increase in that region, although perhaps no one of them alone is completely demonstrative. It is scarcely necessary to point out that the curve has no exact quantitative significance.

On oxidation, either in dye solution or in aerated water after 10–20 minutes in methylene blue 1/30,000–1/50,000 reduced by hydrosulphite, the animal becomes stained throughout, while still completely intact and not appreciably injured. With this procedure it becomes possible to obtain some evidence concerning oxidation-reduction patterns of internal organs, although interpretation of the evidence as regards certain points presents difficulties. Methylene blue is most satisfactory for this purpose because of the depth of color obtainable internally without appreciable toxic effect. Deep staining of the body wall is almost instantaneous in animals removed from the reduced dye to well-aerated water; with less rapid oxidation the gradient pattern appears, except in the head.

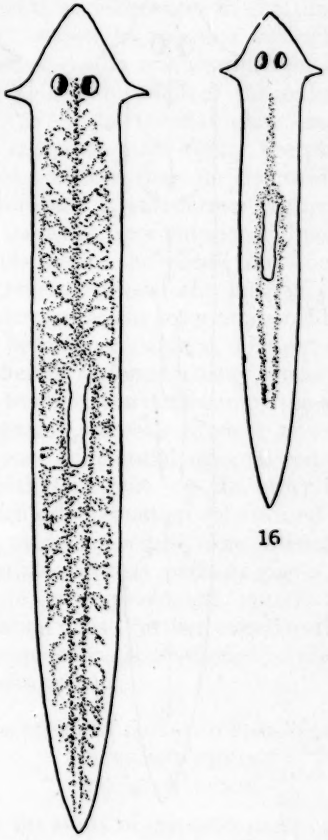
The morphological pattern of the entoderm is, in general, similar to that of *D. dorotocephala* and other planarian triclads; it consists of three main longitudinal trunks or canals, a median prepharyngeal, and two posterior, one on each side of the pharynx and extending to the posterior end. From these trunks, numerous branches extend laterally or anterolaterally in the prepharyngeal region, laterally or posterolaterally in the pharyngeal and postpharyngeal regions. In well-fed animals secondary canals extend longitudinally in lateral regions with interruptions, connecting the tips of the lateral branches from the three main trunks. This entodermal pattern is indicated schematically in Figure 15. After ample feeding and in the earlier stages of starvation the entodermal cells contain protein and fat spherules and granules, which evidently serve as nutritive reserves. Apparently the entodermal epithelium is the only planarian tissue in which any considerable amount of reserves is stored. A histochemical study of intracellular digestion and storage in planarian entoderm by Willier, Hyman, and Rifenburgh (1925) and citations of earlier papers by these authors are of special interest in this connection (see also Hyman, 1946). As the animals decrease in size during extended starvation, these cell inclusions decrease and disappear, and entodermal cells also degenerate. Disappearance of entoderm by degeneration of cells, and perhaps also by decrease in cell size in consequence of loss of the inclusions, progresses from lateral toward median regions and less rapidly from anterior and posterior ends of the main entodermal trunks. In advanced stages of starvation, only the three main entodermal trunks with short diverticula, representing all that remains of the lateral branches, are present (Fig. 16). With continued feeding after such reduction by starvation, entodermal branches gradually grow out again. The entodermal cells as well as the accumulated reserves evidently serve as nutritive material during starvation, and the decrease in size of the animals to a small fraction of the original size indicates that other body cells are also used in the same way. It seems to be very generally true that, in starvation, tissues which are less continuously or less intensely metabolically active are used as nutrition more rapidly than are those more continuously or intensely active. In the past it has sometimes been held that less essential organs underwent reduction in size in starvation more rapidly than did the more essential; this may mean much the same as differences in metabolic activity.

If these views are to some degree correct as regards the planarian, the more rapid disappearance of lateral and terminal entodermal regions in starvation suggests that entodermal metabolic activity decreases from the main trunks laterally and from the middle region just anterior to the pharynx anteropetally and posteropetally. The question of whether entodermal indicator patterns provide any evidence bearing on this point is of interest.

In well-fed animals, brought into aerated water after 10–20 minutes in reduced methylene blue or on aeration of the reduced solution, entodermal oxidation begins laterally, anteriorly, and posteriorly and progresses toward the median plane and from anterior and posterior ends toward the region of junction of the three main trunks. This oxidation pattern is indicated in Figure 17. Both rate and completeness of oxidation depend on the oxygen content of the water or solution. With low oxygen content, only lateral, anterior, and posterior entodermal regions oxidize. With higher oxygen content, complete oxidation may occur in 5–30 minutes, depending on dye concentration, length of time in reduced dye, and degree of aeration.

Reduction begins in the region of junction of the three main trunks and progresses anteriorly and posteriorly and at any given body-level laterally; it is completely opposed in direction to oxidation (Fig. 18). In the postpharyngeal region the short diverticula on the median sides of the two main trunks reduce before the main trunks. More or less differential entodermal reduction usually follows oxidation, even in oxidized dye solution, apparently to a greater extent in animals kept in light on the microscope stage than in those kept in darkness; but it is more or less completely prevented by maintaining the oxygen content of the external medium at a high level.

A feeding with blood clot, a day or two before exposure to the dye, provides the most satisfactory conditions thus far discovered for observing entodermal oxidation and reduction. After the blood feeding, the entire entodermal lumen to the tips of the smallest branches is red. On intracellular oxidation of methylene blue the red color is progressively obscured by blue, and, as reduction occurs, the red

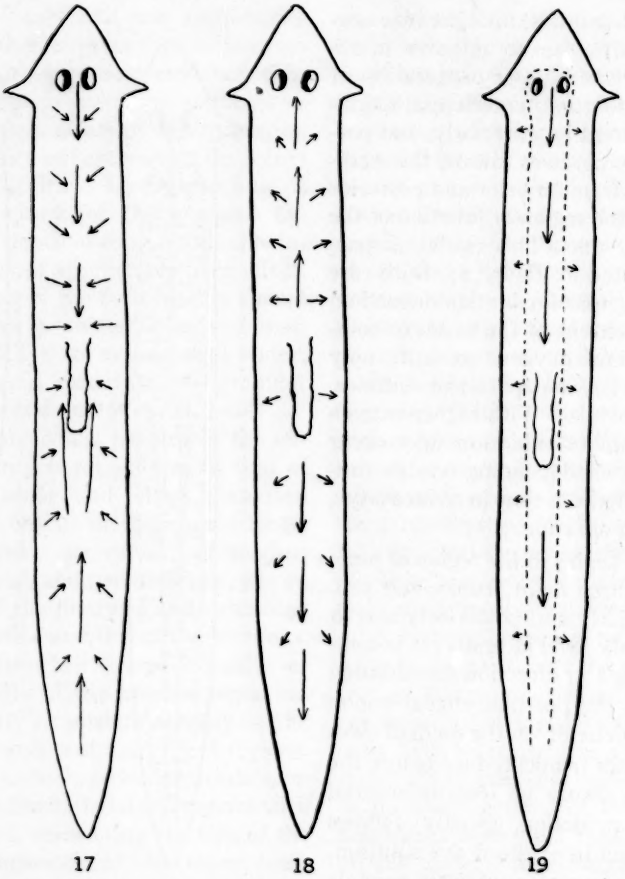


15 16

FIGS. 15 and 16.—The morphological pattern of the planarian entoderm. Fig. 15, well-fed animal. Fig. 16, after several months without food.

reappears progressively. The directions of progress of oxidation and reduction are not affected by the blood cells in the lumen. With this procedure the progress of oxidation can be observed in detail and usually repeatedly in the same animal. Other dyes used have been found less satisfactory than methylene blue for entodermal oxidation and reduction, for various reasons—greater toxicity, less dense color, and consequently less clearly visible differentials, e.g., the red color of partially oxidized Janus green, which is not distinct in the dark entoderm, and the absence of its reduction except in extremely low oxygen.

The pharynx is often protruded through the mouth or through the body wall after irritation by oxidized methylene blue; it undergoes extreme extension and contraction and often separates completely from the body. Intracellular oxi-



FIGS. 17–19.—Oxidation and reduction patterns of planarian entoderm and the median zone. Fig. 17, directions of progress of entodermal oxidation indicated by arrows. Fig. 18, directions of reduction indicated in the same way. Fig. 19, reduction gradient in the median zone; with high oxygen content of the external liquid, the oxidation gradient following 10–20 minutes in reduced methylene blue is in the same direction.

dation and reduction progress from its oral free end toward its normally attached base, i.e., anteropetally as regards the planarian body. It usually undergoes secondary reduction in oxidized methylene blue solution or oxidizes only slightly. The indicators apparently penetrate the pharynx relatively slowly.

One other feature of the internal planarian pattern requires mention. After 10–20 minutes in methylene blue 1/20,000–1/50,000 reduced by hydrosulphite, followed by aeration, a sharply defined internal median zone ventral to the entoderm and extending from the regions of the cephalic ganglia through the entire length of the body usually oxidizes less rapidly than other internal parts and anteropetally or may not oxidize at all if the oxygen content of the external medium is not high. With high external oxygen content, e.g., when animals are brought at once from the reduced solution into well-aerated water, oxidation in the median zone may progress posteropetally from the region of the cephalic ganglia. After complete oxidation of all parts, including the median zone, more or less reduction usually occurs in the median zone in oxidized solution, beginning in the region of the cephalic ganglia and progressing posteropetally. This reduction pattern is indicated in Figure 19. But if the external medium is again deprived of oxygen by hydrosulphite after oxidation of the median zone, reduction progresses from the external surface of the body inward, and earlier reduction of the median zone is less distinct or does not appear. The lateral borders of the median zone coincide with the lateral borders of the longitudinal nerve cords, and the region of the cephalic ganglia constitutes its anterior end. It seems probable that it represents primarily the oxidation-reduction pattern of the nervous system and the region between the nerve cords, where there are transverse commissures.

The period in reduced dye solution, of course, involves more or less complete deprivation of oxygen. With periods of 10 minutes or more, motor activity decreases, but recovery is rapid on aeration. The oxygen available to the internal organs must diffuse in from the exterior, and the internal organs undoubtedly use more or less oxygen. Both the entodermal pattern of oxidation and that of the median zone raise the question whether or to what extent these patterns are determined by differences in the times at which oxygen in amounts sufficient to permit oxidation reaches different internal regions and whether the reduction patterns of the entoderm and median zone represent more nearly the physiological patterns than do the oxidation differentials. If entodermal oxygen uptake increases from anterior and posterior ends toward the middle and from lateral regions toward the median plane, sufficient oxygen to permit oxidation may become available at different times in different regions and may conceivably play some part in determining the oxidation patterns of both entoderm and median zone. Differential internal reduction of methylene blue, on the other hand, particularly reduction in oxidized solution, evidently results from internal differentials and may perhaps give a truer picture of physiological pattern than oxidation does. Further consideration of these questions is postponed to the general discussion.

PATTERN IN ISOLATED PLANARIAN PIECES AND DURING RECONSTITUTION

In the study of reconstitutional oxidation-reduction patterns, 151 pieces were used, at stages ranging from pieces immediately after section to animals with

well-developed head and posterior ends from a week to 2–3 weeks after section. Earlier planarian studies have shown that, in *D. dorotocephala* and *D. tigrina*, head frequency—that is, the frequency of normal and of inhibited degrees of head development in a series of pieces of similar length from animals of similar length with anterior ends at approximately the same body-level—and rate of head development decrease with decrease in length of piece and with increasing distance from the head of the original animals to the level of fission and increase again in the posterior zoöid. The head forms which develop in relation to length of piece and level of body and are also determined by various external depressing agents range from completely normal heads through a graded series of differentially inhibited head forms to completely acephalic forms (Child, 1941, pp. 177–96, 406–11, and citations given there). The series of head forms represents increasing degrees of mediolateral and anteroposterior inhibition of head development. For convenience, five groups of head forms have been distinguished: normal; teratophthalmic, with eyespots approximated to the median plane and pigment areas connected or a single median pigment area with two sensory regions; teratomorphic, with a single median eyespot and cephalic lobes approximated to the median plane anteriorly or a single median lobe; anophthalmic, without eyespots or cephalic lobe but with a single median ganglionic mass; acephalic, head and anterior outgrowth of new tissue completely absent. While pieces that will remain acephalic can be distinguished from others rather early in reconstitution, the particular type of head which will develop on pieces below a certain length can be identified only in later stages of reconstitution. Essentially the same series of head forms in similar relation to length of piece and body-level appear in the California planarian species. Since the inhibited head forms develop more slowly than do the normal forms, it was thought possible that differences in rate of oxidation and reduction of indicators might appear in relation to degrees of head development. Extended investigation of this point has not been possible, but some evidence of such a relation has been obtained.

PATTERN IN RECONSTITUTION OF NORMAL ANIMALS

The typical reconstitutional pattern, as it appears in pieces with anterior and posterior cut ends and from which complete individuals with normal heads develop, e.g., piece *A* or *B* of Figure 20, is as follows: As soon after section as the piece can be brought into Janus green completely reduced by hydrosulphite, the rate of intracellular oxidation in the body wall is distinctly more rapid at the anterior cut end than elsewhere and decreases posteropetally for a short distance. At the posterior cut end a slight increase in rate of oxidation appears, distinctly less than at the anterior end; and the anteropetal gradient of decrease is shorter than that at the anterior end. Evidently, the activation or stimulation following section is greater and extends farther at the anterior, than at the posterior, end. The oxidative color change is most conspicuous in the body margins and ventrally; in the margins the color is seen through a double layer of cells, elsewhere through a single layer; but the activation is apparently greater ventrally than dorsally. The part of the piece between the two activated regions shows no distinguishable change in the posteropetal gradient pattern. In Figure 21 the shading represents the regions in which the pattern is altered following section; the arrow indicates the gradient in other

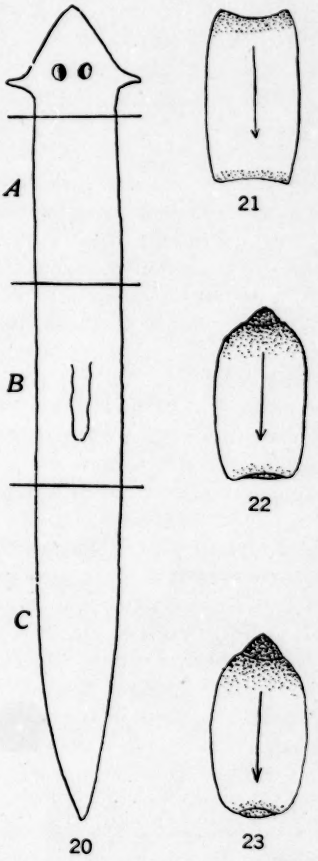
parts of the piece. Other dyes and the indophenol reaction show the same pattern, and after very slight staining by oxidized Janus green the reduction pattern is similar. After rapid and deep staining by oxidation of reduced methylene blue, reduction may be delayed in the activated regions. Evidently the effect of section on tissues adjoining the cut ends differs greatly at the two ends.

Attempts were made to determine whether the cut ends of the nerve cords at the open ends of pieces show any difference from other tissues. It is perhaps worth noting that in several cases two regions of slightly more rapid indophenol reaction were visible in the regions where cut ends of the cords would be; that they were the nerve cords is not regarded as certain. Nothing definite was observed with the dyes.

Entoderm and parenchyma usually protrude from cut ends and soon disintegrate in water; later intact entoderm appears to be slightly retracted from the cut end. It has not been determined with certainty whether oxidation and reduction are increased in the entoderm following section; in some cases a slight increase has seemed to be visible at some time within an hour after section.

The new tissue which appears at the anterior end shows a higher rate of oxidation than any other part of the piece and higher than the rate in the old tissue immediately following section. In the old tissue of the anterior region the rate decreases posteropetally. At the posterior end, development of new tissue is less rapid, oxidation is also less rapid and extends a shorter distance anteriorly in the old tissue. The oxidation pattern in an early stage of reconstitution is indicated in Figure 22 and in a somewhat later stage in Figure 23.

The new tissue is so highly susceptible to the toxic effects of the indicators that it usually reduces less rapidly than do other parts of the piece; but, with very slight staining by oxidized Janus green or after slight indophenol reaction, it reduces earlier than other parts do. With methylene blue, even after slight staining, it usually reduces less rapidly than the rest of the piece does. The posterior new tissue is much less susceptible and, unless deeply stained, reduces somewhat



FIGS. 20–23.—Oxidation-reduction patterns in planarian reconstitution. Fig. 20, outline indicating *A*-, *B*-, and *C*-pieces. Fig. 21, indicator pattern immediately after section, with higher rate of oxidation at anterior, than at posterior, cut end. Figs. 22 and 23, patterns at two early stages of reconstitution.

earlier than does adjoining old tissue. With further development of the head, the pattern of oxidation and reduction at the anterior end of the piece approaches that of the fully developed head. The difference between anterior and posterior ends persists. In later stages, as the posterior end approaches complete development, the increase in rate characteristic of intact animals appears at its extreme tip.

OXIDATION IN RELATION TO BODY-LEVEL AND HEAD FORM

The limited amount of material of the California planarian available made it impossible to use large numbers of pieces in this group of experiments. In all cases the pieces to be compared were in the same cell, containing slightly more than 0.5 ml. of completely reduced Janus green, with air excluded and with frequent movement of the slide to insure uniformity in the solution. After section of animals 12–15 mm. in length into three pieces—*A*, *B*, and *C* of Figure 20, the *C*-pieces representing approximately the posterior zoöid region—the increase in rate of oxidation of the dye within the first 15 minutes was greater in the *A*- and *C*-pieces than in the *B*-piece in the four series of pieces tested. In two of these series the *C*-piece was more rapid than the *A*-piece. In early stages of reconstitution, 3 days after section, oxidation in the developing anterior new tissue was more rapid in *A* than in *B* and probably more rapid in *C* than in *B*, though the difference was at best slight and was not regarded as beyond question; this group included three lots of pieces, all giving the same result. Secondary reduction to colorless after oxidation to red and disintegration of the anterior new tissue occurred first in *A*, then in *C*, and last in *B*. In three comparisons, 15–30 minutes after section, of pieces 2–5 mm. in length with pieces 1 mm. or less, with anterior ends at approximately the same level, oxidation was clearly more rapid at the anterior ends of the longer pieces.

In three comparisons of *A*-pieces with normal heads and pieces from the middle body region with teratophthalmic heads, all at early stages of development of eyespots, 5 days after section, all three showed distinctly more rapid oxidation in the normal heads. In two comparisons of *A*-pieces with developing heads and very short acephalic pieces from the middle body region, both 3 days after section, the anterior new tissue of the *A*-pieces showed the usual rapid oxidation; in the acephalic pieces, anterior increase in oxidation was very slight and was limited to the region of wound closure at the extreme tip. Except for the uncertainty as regards differences in rate of oxidation of the anterior new tissue in comparisons of *B*- and *C*-pieces, 3 days after section, these few data agree completely with the results of reconstitution as regards rate of head regeneration and occurrence of inhibited head forms in pieces from different body-levels and of different lengths.

It has long been known that heads isolated by section a short distance posterior to the eyspots may remain alive for weeks. The wound closes rapidly, but no development of more posterior parts or of new tissue beyond that directly concerned in wound closure takes place. Moreover, there is only a mere trace of increase in rate of oxidation in the region of wound closure (Fig. 24). Earlier work has shown that, in short pieces of *D. dorotocepha a* just posterior to the head, it can be determined experimentally whether posterior ends develop or are completely absent. In other words, the scale of organization in such pieces can be decreased or increased. The scale of organization has also been altered in

other pieces and in other organisms (Rulon and Child, 1937; Child, 1941, pp. 343–57). The almost complete absence of activation at the posterior ends of pieces like Figure 24, although the same body-level, when at the anterior end of a piece, undergoes rapid and great activation and develops into a head, suggests that dominance of the head rather than lack of capacity to develop is concerned. According to this view, the cells at the posterior end of the head piece are prevented from undergoing the change in a condition which expresses itself in activation and development. Under the dominance of the high gradient levels anterior to them, they continue to function as particular body-levels and cannot give rise to a new gradient pattern. If they are at the anterior end of a piece, they represent the highest gradient level in the piece and are relatively or completely independent of other parts.

ENTODERMAL OXIDATION-REDUCTION PATTERNS DURING RECONSTITUTION

The deep coloring of the entoderm on oxidation after exposure to reduced methylene blue (p. 339) provides a basis for bringing to light some of the entodermal changes in relation to reconstitution. In pieces from the prepharyngeal region, e.g., *A*-pieces (Fig. 20), no clearly distinguishable change has been observed in the anterior entoderm adjoining the regenerating head. There is perhaps a slight increase in rate of oxidation, but comparison with intact animals shows little difference. The more rapid oxidation indicated by deeper shading in the anterior entoderm of Figure 25 differs little, if at all, from the differential in intact animals. Reconstitution in these *A*-pieces involves development of a new pharyngeal and postpharyngeal entoderm. The new pharynx develops ventral to the entoderm and evidently determines division of the median trunk into the two trunks on the two sides of the pharynx. There is apparently some retraction of entoderm from the posterior cut end following the protrusion and disintegration before closure of the wound. The two postpharyngeal trunks are formed by growth posteriorly of the two trunks from the new pharyngeal region; they show an increase in rate of oxidation, as indicated by shading in Figure 25. Further progress of entodermal oxidation in these pieces is essentially like that in intact animals (p. 339). The progress of reduction is opposite in direction to that of oxidation, as in intact animals, the median region immediately anterior to the new pharynx oxidizing less rapidly and reducing more rapidly than other parts.

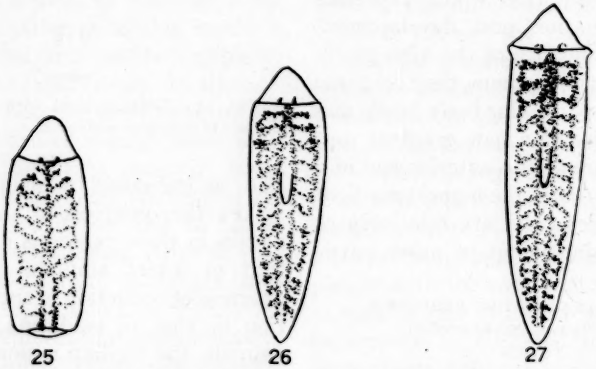
FIG. 24.—Isolated head with almost complete absence of posterior activation.

In *B*-pieces (Fig. 20), which include the old pharynx, the anterior and posterior entodermal regions become respectively more anterior and more posterior, as regards rates of oxidation. They are regions of relatively low rate in intact animals (Figs. 17 and 20), and in reconstitution they show distinct increases in rate, corresponding to their change from regions near the middle to anterior and posterior terminal regions.

Entodermal changes in the *C*-pieces (Fig. 20), which, in the longer animals, represent approximately the posterior zoöid region, are perhaps the most interesting. In these pieces the anterior entodermal region—a region with very low

rate of oxidation in intact animals, since it is near the middle of the body—becomes the terminal anterior entoderm. The two longitudinal trunks characteristic of the postpharyngeal region unite posteropetally to become a single median trunk. Two stages of these changes are indicated in Figures 26 and 27. In the new prepharyngeal region, which develops from the more anterior part of the original postpharyngeal region, the change in entodermal condition and structure is greater than elsewhere. In the intact animal, the rate of entodermal oxidation decreases anteropetally throughout the whole length of the region, which in reconstitution becomes the entoderm of an entire animal. With the determination of a prepharyngeal region and a shorter postpharyngeal region, the new pharynx develops in what was originally approximately the middle of the postpharyngeal region, and the rate of entodermal oxidation increases anteropetally and posteropetally from it. The rate of reduction decreases in the same directions.

The impossibility of controlling nutritive conditions adequately and the difference in general physiological condition between individuals resulting from reconstitution and intact animals like those from which the pieces were taken renders comparisons of rates of entodermal oxidation in the two of doubtful significance. Consequently, it remains uncertain whether or not there is actual decrease in rate of entodermal oxidation in the new pharyngeal region. In the body, in general, the rate of oxidation and of reduction increases during reconstitution; the resulting animal is physiologically younger than the original animal. Moreover, it has been observed repeatedly that in the later stages of reconstitution (e.g., Fig. 27) the whole prepharyngeal regions of *C*-pieces, which were originally the more anterior parts of postpharyngeal regions, reduce distinctly more rapidly than do the new postpharyngeal regions, which have remained postpharyngeal. Presumably, the part of the postpharyngeal entoderm which has become prepharyngeal has undergone greater change in condition than has the part which has remained postpharyngeal.



FIGS. 25–27.—Changes in entodermal pattern in relation to reconstitution. Fig. 25, *A*-piece, with increased rate of oxidation in the new postpharyngeal trunks growing posteriorly. Figs. 26 and 27, earlier and later stage of *C*-piece; part of the originally postpharyngeal entoderm becomes prepharyngeal, acquires the pattern of prepharyngeal entoderm, and, with progress of reconstitution, the entoderm of the piece—all originally postpharyngeal—acquires the oxidation-reduction pattern of an entire animal.

DISCUSSION

In the reconstitution of hydra pieces there is no regeneration in the strict sense, that is, outgrowth of new, more or less undifferentiated tissue from the surface of section, followed by differentiation of the outgrowth. Cut ends of the pieces close rapidly, and reconstitution consists in direct reorganization and redifferentiation of parts or of the whole of the piece. Reconstitution in the planarian consists in part of regenerative outgrowth and differentiation of the new tissue and in part of reorganization and redifferentiation of other parts. In spite of these differences, not only are the direct effects of section on the patterns of oxidation and reduction of indicators essentially similar in the two forms, but the redifferentiating tissue of hydra shows the same alteration of oxidation-reduction pattern as do the regenerating parts and the redifferentiating parts adjoining them in the planarian. In both forms there is establishment of a new gradient pattern, rendered directly visible by an increase in the rate of oxidation and reduction extending with a decrement over a variable distance from the closed end of the piece in hydra and from the regenerating part into the "old" part undergoing reorganization in the planarian. These changes indicate a marked stimulation or activation, extending a greater or less distance from the level of section in both forms.

In the hydra this activation is much greater at the distal ends of pieces and in the planarian, at the anterior ends; and in both forms it extends over a greater distance than at the proximal or posterior ends of pieces. In extremely short pieces, not specially considered in this paper, the difference at the two ends is less or not distinguishable. The difference is an expression of the original longitudinal gradient pattern and the dominance which results from it. Any particular body-level of hydra or the planarian undergoes much greater activation when it constitutes the distal or anterior end of a piece than when it is proximal or posterior. In both forms the activation resulting from injury is temporary and decreases or disappears with closure of the wound; the later activation, which persists and increases in reconstitution, results from isolation from the dominance of more distal or anterior regions. It precedes morphogenesis, and the evidence at present available indicates that it is the initiating factor in the reconstitution of more distal or anterior parts from the distal or anterior ends of pieces. It represents primarily the imposition of a new gradient pattern with higher gradient levels on the piece.

At the proximal or posterior ends of pieces there is no complete isolation from more distal or anterior regions; and the slight, often almost inappreciable, activation in earlier stages of reconstitution is wholly or largely a direct effect of injury. With the development of proximal or posterior parts, some activation occurs, but even the new regenerating tissue at the posterior end of the planarian piece shows relatively little increase in rate of oxidation and reduction of indicators.

The hydra tentacle and the planarian head show the same difference at opposite cut ends. The tentacle undergoes activation at the distal cut end after removal of a part and reconstitutes distally; proximally there is little or no activation and no reconstitution. In the planarian head activation and reconstitution occur anteriorly, not posteriorly.

There is nothing in the planarian body corresponding to the stalk of *Pelmatohydra*. There is scarcely a trace of activation at the distal cut end in a piece of

stalk, and reconstitution of more distal parts does not occur under the usual conditions, although the piece may live until reconstitution is complete in pieces from the body. Activation, as shown by increase in the rate of oxidation and reduction of indicators at distal and anterior ends of pieces, appears to be essential to reconstitution of more distal or anterior regions. Whether or not the stalk of *Pelmatohydra* is more highly differentiated than the body, it is apparently more stably differentiated.

As already noted (p. 341), the patterns of oxidation and reduction of methylene blue in the planarian entoderm raise certain questions. The significance of the progress of entodermal oxidation from anterior and posterior ends and from lateral to median regions and the progress of reduction in opposite directions is uncertain. In oxidation of the reduced dye already present in the entodermal cells, the oxygen gradually diffuses in from the exterior. The question arises whether the oxidation pattern indicates a decrease in oxidase activity and oxygen uptake or an increase in the direction of progress. It was noted above (p. 338) that in starvation the entoderm disappears progressively from anterior, posterior, and lateral regions, i.e., in the same direction as the progress of oxidation of methylene blue. It was also noted that parts which are relatively inactive, at least preceding the starvation period, decrease in size or disappear earlier or more rapidly than do those more continuously or intensely active. There may perhaps be a graded increase from peripheral to central entodermal regions in digestive activity, as distinguished from intracellular storage of nutritive material; and the progress of entodermal disappearance in starvation may be an expression of this differential.

If oxidative activity increases from peripheral toward central entodermal regions, oxygen diffusing from the exterior may become sufficient to permit oxidation of the dye earlier in the less active, than in the more active, parts. Moreover, the planarian body increases in dorsiventral thickness from lateral to median regions and slightly from anterior and posterior regions toward the middle. In short, it appears possible that the pattern of entodermal oxidation of methylene blue in the intact planarian may represent, at least in part, a differential in oxygen diffusion from the exterior to the entoderm rather than only a differential in oxidative activity; it may perhaps even be the reverse of the gradient pattern of oxidation. It has been found that, even in the single cell of *Paramecium*, other ciliates (Child, 1945), and various other organisms, intracellular oxidation of reduced indicators progresses posteropetally with rapid and considerable oxygen increase in the external medium and anteropetally when the increase is slow and relatively slight. In the latter case the oxygen available is sufficient to permit dye oxidation earlier in regions of less oxygen uptake than in other regions. In these cases available oxygen, not oxidase or oxygen uptake alone, becomes the factor determining the gradient pattern of dye oxidation; and this gradient is the reverse of that appearing with higher oxygen content of the external medium. It is possible that the oxidation pattern of the planarian entoderm is of this character.

The question also arises whether, or to what extent, the entodermal dye-reduction pattern, with reduction progressing anteriorly, posteriorly, and laterally from the middle region just anterior to the pharynx, represents a real physiological pattern. However, it appears difficult to interpret the reduction pattern in terms of differential oxygen supply. In reduced

dye solution there is probably more rapid oxygen loss from the thinner lateral, than from the thicker median, regions; but median regions reduce more rapidly than lateral. Moreoever, differential entodermal reduction occurs to a greater or less extent even in oxidized methylene blue solution or in aerated water. Decrease in oxidative activity anteriorly, posteriorly, and laterally will account for the entodermal reduction pattern, increase in the same directions will not.

In animals kept without food for 36 and for 50 days there is much less differential in entodermal oxidation of methylene blue; it does progress from the tips of the branches but may be complete in less than a minute instead of in 5 minutes or more. This suggests the possibility that the entodermal oxidation pattern may be correlated with a physiological differential more or less dependent on presence of food. Still another possibility is that there is a gradation from central to peripheral regions of one metabolic system into another different in character, also perhaps dependent to some extent on the presence of food. In short, this examination of the possible interpretations of entodermal indicator patterns in the planarian does not lead to definite conclusions, at least as regards the oxidation pattern; but it may perhaps serve to call attention to some of the difficulties involved in determining the significance of indicator pattern in internal organs.

Some of the changes in entodermal patterns of oxidation and reduction in relation to reconstitution also raise questions. In the prepharyngeal *A*-pieces the only marked change is the increase in rate of oxidation in the entoderm growing posteriorly to form the two postpharyngeal trunks (Fig. 25). Increase in rate of reduction in these growing regions has not been observed but probably occurs. In the *C*-pieces from the postpharyngeal region of the parent-animal the changes are greater. The entoderm of the new prepharyngeal regions of these pieces originally oxidized much less rapidly and reduced more rapidly than did more posterior levels. During reconstitution it undergoes a great increase in rate of oxidation of methylene blue, with a posteropetal gradient instead of the anteropetal gradient originally present, and it reduces anteropetally and laterally from the region just anterior to the new pharynx instead of posteropetally, as originally. In other words, it becomes prepharyngeal entoderm physiologically as well as morphologically. In the later stages of reconstitution the entire entoderm of the new prepharyngeal region reduces more rapidly than that which is still postpharyngeal, probably because it has undergone much greater change physiologically and morphologically in becoming prepharyngeal entoderm and in the union of the two trunks into a single median trunk. In the *B*-pieces, which contain the old pharynx, the anterior part of the prepharyngeal entoderm becomes more "anterior," with an increase in rate of oxidation and a decrease in rate of reduction; and the posterior part of the postpharyngeal entoderm becomes more "posterior" with similar changes. In *A*-, *B*-, and *C*-pieces entodermal oxidation-reduction patterns gradually become like those of a complete individual smaller than the parent-animal. These changes in entodermal oxidation-reduction patterns during reconstitution suggest that physiological factors rather than merely differentials in available oxygen are concerned in oxidation, as well as in reduction patterns of planarian entoderm. Whatever the interpretation, it is evident that the planarian entoderm shows definite oxidation-reduction patterns with certain definite changes in relation to reconstitution.

The planarian median zone of more rapid methylene blue reduction is believed to represent primarily the cephalic ganglionic region, the longitudinal nerve cords, and the region between them with transverse commissures. Reduction is more rapid in the region of the cephalic ganglia than in other parts of the body and progresses posteropetally; at any particular body-level it is more rapid than in other parts at that level. With high oxygen content of the external liquid, oxidation of the median zone may progress posteropetally; with less oxygen it is usually anteropetal. In earlier observations on methylene blue reduction in a planocerid polyclad, it was found that, when the animals were stained throughout the body, reduction was most rapid in the cephalic ganglia and progressed in all directions from them, most rapidly posteropetally (Child, 1941, p. 119).

From these data on hydra and the planarian it appears beyond question that intracellular reduction and oxidation of indicators bring to light certain features of physiological gradient patterns and the changes which they undergo during reconstitution, although some uncertainties arise as regards the significance of entodermal patterns.

SUMMARY

1. Alterations in oxidation-reduction indicator patterns in reconstitution of isolated pieces of hydra and a planarian are, in general, very similar, except as regards certain features associated with entodermal differences in the two forms.

2. In isolated pieces an increase in rate of oxidation and reduction of indicators, greater at distal or anterior than at proximal or posterior ends, follows section immediately as a direct effect of injury. This is replaced or followed by a gradient of increased rate, extending with a decrement from the distal or anterior cut end for a certain distance. At the proximal or posterior cut end there is much less evidence of activation and a much shorter and less sharply defined gradient.

3. The differences at the two ends of pieces are features—first, of the longitudinal gradient pattern of the parent-animal and the piece as a part of it and, second, at the distal or anterior end—of the isolation from the dominance of more distal or anterior regions.

4. The alteration at the distal or anterior end constitutes imposition of a new longitudinal gradient pattern on the piece. This precedes any evidence of morphogenesis and becomes the locus of development of distal or anterior regions of the new individual.

5. Reconstitution of proximal or posterior parts is determined by relation to other parts of the piece and involves only slight changes in oxidation-reduction patterns.

6. In the intact planarian the entodermal oxidation pattern is the reverse of the reduction pattern. In reconstitution the entodermal patterns undergo alteration corresponding to the alterations in regions of the body determined in different parts of the piece during reconstitution. In pieces from different body-levels, different parts of the entoderm of the parent animal attain the oxidation-reduction pattern of an entire individual.

7. The region of the cephalic ganglia reduces methylene blue more rapidly than do other parts of the body when the animals are deeply stained throughout by intracellular oxidation of the reduced dye; and reduction progresses posteropetally from the ganglionic region along the nerve cords and the region between them: at any particular body-level it is more rapid than in other parts at that level.

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PATTERNING OF CENTRAL SYNAPSES IN REGENERATION OF THE OPTIC NERVE IN TELEOSTS[1]

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IT HAD been well established by 1925 that the retina of the grafted adult urodele eye undergoes degeneration except for its ciliary portion, that a new retina is regenerated from this surviving ciliary margin, and that the new optic nerve which develops from the regenerated retina is capable of re-establishing connections with the brain (Fujita, 1913; Griffini and Marcho, 1889; Kolmer, 1923; Uhlenhuth, 1912). Whether the regenerated optic-nerve connections were capable of function, however, was still at that time a matter of controversy. Early reports, emanating from Przibram's laboratory, of visual recovery in many vertebrates, including rats, particularly the claims of Koppanyi (1923), were found to have been based on inadequate tests of vision and later came to be regarded generally as unreliable (Keeler, 1929; Stone and Zaur, 1940).

It was Matthey in 1925 and 1926 who first furnished indisputable evidence of the recovery of vision in adult urodeles after eye transplantation and also after intracranial resection of 1–2 mm. of the optic nerves. Matthey's animals would turn and swim toward a moving worm confined within a glass vessel placed within the animals' aquarium and would then try to snap at the worm through the glass. They would respond similarly toward a finger moved just above the surface of the water but not touching it. Also they would pursue and try to strike through the glass at a piece of liver moved about in the air outside the aquarium walls. These reactions were said to be entirely comparable to those of a normal animal and were absent in a blind animal. Matthey's results were later repeated and confirmed by Stone and others in this country (see Beers, 1929; Stone and Zaur, 1940).

From Matthey's description of visual

[1] The experiments were conducted at the Lerner Marine Laboratory of the American Museum of Natural History, at Bimini, British West Indies. The work was aided by the generous technical assistance of Dr. C. M. Breder and was supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

recovery it could be inferred not only that the regenerating fibers were successful in establishing functional connections in the brain but, furthermore, that the central relations were formed with a sufficient degree of orderliness to permit discrete perception and accurate localization of small objects in space. This was particularly remarkable because nerve regeneration under such conditions is attended by extreme distortion of the normal intraneural fiber pattern. Matthey himself observed that the regenerating fibers failed to restore the orderly fascicular pattern of normal ontogeny and that there was a tendency for some of the optic fibers to become entangled in neuromas and for other stray fascicles to become lost in atypical pathways.

That the recovered vision had, nevertheless, been adaptive, despite the observed disorder among the regenerated fibers would suggest either that (*a*) the fibers had managed to untangle and sort out in an extraordinary manner after reaching the brain centers or else that (*b*) some functional process of adaptation, like learning or conditioning, was instrumental in the adjustment of the central connections. The latter possibility seemed to be favored by the inclusion among Matthey's animals of two cases in which, after resection of the optic chiasma, the regenerating nerves had connected atypically with the ipsilateral, instead of the contralateral, brain center and also by the inclusion among the recoveries described by Stone and his students of cases in which the eyeball had been rotated through 90° and 180°. Presumably, it could be only by means of some process akin to learning that normal vision could be recovered in the face of anatomical rearrangements of this kind.

More detailed analysis of the functional aspects of this problem, however, has since revealed (Sperry 1942–44) that rotation of the eye causes definite and marked symptoms of inverted vision and that, in contradiction to the above, the same type of abnormal vision is restored after optic-nerve regeneration with the eye rotated. It has likewise been found that, when the regenerating optic nerves are intentionally made to connect to the ipsilateral optic centers, the recovered visual responses are clearly misdirected to the contralateral side (Sperry, 1945*a*). Apparently, the earlier failures of Matthey, Stone, and others to observe these functional abnormalities may be attributed to the fact that they did not include optokinetic reactions among their tests of recovery and that they were not considering the neurological problems involved and therefore overlooked the misdirection of localizing responses that were present in their animals.

Further investigation of the functional aspects of optic-nerve regeneration in conjunction with eye rotation, contralateral transplantation of eyes, cross-union of optic nerves, and optic-lobe lesions in urodeles and also in larval and adult anurans of a number of different species (Sperry, 1942–45*a*) has made it clear that the organizational forces involved in the adjustment of the central synaptic associations are quite independent of functional adaptation. Apparently, the developing retinal field undergoes a polarized differentiation, as a result of which the ganglion cells of different retinal loci and the optic fibers arising from them become biochemically specified. The formation of central synapses would seem to be regulated by selective affinities between the different types of optic fibers and the various kinds of central neurons.

The results of optic-nerve regeneration, reinforced later by similar findings in regeneration of other nerves and cen-

tral-fiber tracts (Sperry, 1945*b*–48*b*), have furnished strong evidence for a general biochemical-affinity theory of the ontogenetic organization of synaptic connections. It is only in the amphibians, however, that this type of phenomenon has so far been observed. It has therefore become important to find out whether or not synaptic formation is regulated on a similar basis in other classes of vertebrates. This paper is the result of an effort to extend to teleosts the experiments on optic-nerve regeneration. Functional regeneration of the optic nerve in fishes apparently has not been reported in previous experiments, even where the nerve has been sectioned without damaging circulation (Matthews, 1933). There were encouraging indications, however, in the report of Ask and Andersson (1927) and in unpublished observations of Rasquin that the teleost optic nerve might possess some capacity for functional regeneration. The general plan of the following experiments is the same as that employed in earlier studies on eye rotation and optic-nerve regeneration in amphibians.

MATERIAL AND METHODS

The fishes used were of various species as designated below and were collected in March around the island of Bimini in the West Indies. They were maintained in the laboratory in groups of approximately ten in aquaria 60×30×30 cm. with running sea water at 24°–29° C. and were fed every other day. The animals were anesthetized in a solution of 1.2 per cent urethane in sea water and were then wrapped in damp cleansing tissue and operated upon out of water with the aid of a dissecting microscope. The only other instruments used were fine scissors and watchmaker's forceps.

In order to rotate the eyes, all the extrinsic ocular muscles were extirpated either through a dorsal incision into the orbit in *Bathygobius soporator* (Cuvier and Valenciennes) and *Canthigaster rostratus* (Bloch) or through a complete circular incision around the conjunctiva in all other species. The eyeball, thus freed except for the intact optic nerve and associated blood vessels, was then rotated through 180° on the optic axis and allowed to set a few moments before the fish were returned to water. Only one eye was rotated. The contralateral optic nerve was sectioned, and a segment of it was excised to prevent its regeneration. When it became evident that further precaution in this regard was necessary, the unrotated eye was completely excised in all the *B. soporator*, and the optic nerve in the other species was again severed and partially excised 3 weeks after the initial operation.

The optic nerve was sectioned through a short dorsal incision in the conjunctiva. The eyeball was rolled downward to make the optic nerve accessible from the dorsal approach. The nerve was pinched repeatedly with forceps until complete division of the nerve substance was visible. In many cases the outer sheath of the nerve, which is tougher than the nerve proper, was left partially intact to help guide the regenerating fibers. When optic-nerve division was performed in conjunction with eyeball rotation, the rotation was always carried out prior to nerve division.

The types of reactions used in estimating the quality of visual performance and recovery included (*a*) the optokinetic responses induced by rotation of a striped drum around the animals in the horizontal plane; (*b*) the spontaneous optokinetic reactions which have been found to follow eye rotation (Sperry, 1943*a*); (*c*) avoidance of obstacles, such as the aquarium fixtures, large shells, and other fish, while swimming at high speed; (*d*)

escape reactions involving flight away from approaching objects, usually a small net with a frame 10×12 cm., and location of shelter; and, lastly, (*e*) positive localizing reactions, such as the seizing of small particles of food floating in the water or sinking to the bottom and the pursuit of other fish. The extent to which any one of these different reactions could be used to test vision varied considerably in different species. By employing the entire battery of tests, however, it was possible to obtain criteria of visual performance in all species which were adequate for the purposes of the experiments. In general, the optokinetic response was found to be less satisfactory than in amphibians and accordingly was relied upon to a lesser degree.

In some of the animals, rotation of the eye was attended by loss of vision, due probably to the cutting-off of circulation or to compression of the twisted optic nerve. These animals were discarded from the experiments, as were also some others that became infected and some that expired before vision was recovered after nerve division. Losses from such causes were negligible among *B. soporator*, a hardy tide-pool form, but were approximately 50 per cent among the other species.

The animals with regenerated nerves were subsequently prepared for microscopic examination by the Bodian silver proteinate method.

RESULTS

180° ROTATION OF THE EYE

The right eye was successfully rotated without loss of vision, and the left optic nerve was excised in eight *B. soporator*, two *Sparisoma radians* (Cuvier and Valenciennes), two *Abudefduf saxatilis* (Linnaeus), two *Pomacentrus leucostictus* (Müller and Troschel), and one *C. rostratus*. These fishes ranged between 4 and 10 cm. in length. In general, their subsequent behavior indicated that the reorientation of the retina had caused an illusory 180° rotation in the appearance of the visual field. The evidence of this optic inversion varied somewhat in detail in the different species.

The most striking effect of the shift in retinal orientation was the forced circling activity which it caused. The *P. leucostictus* turned continuously thereafter around a fixed spot close to the bottom of the aquarium, as a result of which they fanned out deep excavations in the sand in which they continued to rotate incessantly day after day. Their circling ceased only at night and for brief moments when they bumped against the aquarium walls, fixtures, or other fish. Food impaled on a fine wire was presented from the blind side in a position such that they turned directly into it. Under these conditions food was regularly taken after the first week with little interruption of the circling activity.

The *A. saxatilis* also circled continuously, but not in a fixed spot like the *P. leucostictus*. They swam in circles of a diameter approximately five times their total length at varying depths, keeping away from the bottom and walls of the aquarium. The *S. radians* rested most of the time on the bottom under the shelter of seaweed; but, whenever these fish were aroused from the bottom, they whirled wildly at very high speed in circles of a diameter roughly that of their total length. The circling movements of *B. soporator* were usually restricted to turning the head with the body held in place by the modified ventral fins by which these fish cling to the bottom and even to vertical glass walls. Not infrequently, however, they darted and twisted spasmodically in complete circles, sometimes at high speed. Occasionally, backward

swimming, tilting, side-slipping, and various peculiar gyrations were also exhibited in the various species, indicating illusory movement of the visual field in other planes.

Optokinetic responses induced by a revolving drum were made in the direction opposite from normal by all species. Unlike the amphibians, the fish, in most cases, responded to movement of the visual field in either direction, even though one eye was blind. The spontaneous circling reactions described above were also made in both directions except in the *P. leucostictus*, which always circled toward the blind side. The responses tended to be stronger and more frequent toward the blind side, but one *A. saxatilis* circled continuously toward the seeing side. Getting started toward one side or the other seemed to reinforce further turning toward the same side.

Both the imposed and the spontaneous optokinetic reactions were accompanied by nystagmic movements of the left blind eye, the extrinsic muscles of which had been left intact. The slow phase of the movements corresponded in direction with that of the body movement. Nystagmus was extremely marked in *S. radians*, slight in *B. soporator*, and of intermediate degrees in the other species and was correlated, apparently, with the relative amount of compensatory eye movement to which they are inherently subject under normal conditions. The nystagmus frequently continued independently when head and body movements were inhibited and when the fish were resting on the bottom. These independent eye reactions were particularly pronounced in *S. radians*.

Directional reversal of responses toward and away from objects were obscured in part by the persistent forced circling. The *S. radians*, for example, circled so rapidly whenever they stirred that any localizing reactions were impossible. Erroneous localization was evident, however, in some of the other species. The *A. saxatilis*, *C. rostratus*, and *P. leucostictus* circled upward when a small net approached them from above and downward when it approached from below. The *B. soporator* darted forward when the net approached from in front, and they turned back toward the net when it approached from the rear. Often the fishes swam directly into the net under these conditions. Conversely, the *A. saxatilis* and the *P. leucostictus*, after they had become accustomed to the feeding procedure, circled upward when food was presented below them and downward when it was offered above. The *B. soporator* started forward when the lure was offered to the rear, turned backward when it was presented in front, and occasionally snapped downward when it was presented close above them.

One of the *P. leucostictus* assumed an inverted color phase characteristic of this species at night or after it has been blinded by optic-nerve section. All the *B. soporator* became extremely dark, in marked contrast to the light color of control and unoperated cases. Two extra cases with both eyes rotated also became very dark. Brief attempts to demonstrate adaptation to overhead lighting instead of to the bottom were unsuccessful, and the consistent change to the dark-color phase following eye rotation in this species remained unexplained. It has been observed that the color changes of these fishes are considerably influenced by factors other than that of the background (Beebe, 1931).

Mock operations were performed on a series of control cases, including two *B. soporator*, two *A. saxatilis*, and two *P. leucostictus*. The operations were similar in every way to those performed on the experimental group, except that the eye

was left in its normal orientation. The mock operations, however, failed to produce any of the symptoms of reversed vision which followed rotation of the retinal field in the experimental group.

Effect of experience.—The cornea of the *C. rostratus* became increasingly cloudy and opaque, beginning on the 2d day after operation. By the 6th day after operation this animal was no longer circling or showing other signs of vision, and it died 3 days later. All the other cases survived the first 14 days after operation with the eye in good condition. There was little or no evidence of any re-education at this time. The free-swimming species circled a trifle more slowly than during the first 2 days, and the circling seemed to be done with less intensity and in a more automatic and relaxed fashion. When excited, however, they circled as rapidly as at the beginning, and they still exhibited directional reversal of responses to food objects and a small net.

One *S. radians* was found dead on the 15th postoperative day, without having shown any signs of readjustment. During the next 3 days the two *A. saxatilis* became weaker and expired, apparently from insufficient food intake plus the excess activity involved in their continuous circling. There were some signs of adaptation in the latter two cases, in that other responses could be superimposed upon the forced circling movements to a greater degree and more effectively than at the beginning. They continued to show directional reversal, however, in their attempts to escape a net or to approach food particles. The second *S. radians* survived 22 days. During the last 6 days it had ceased to swim in circles. Its swimming at this time was erratic and unsteady, and efforts to avoid an approaching net remained inefficient. Locomotion was greatly improved, however, over that of the first 2 weeks, when the rapid forced circling had predominated. The unrotated blind eye continued to exhibit nystagmus.

At 24 days after operation the two *P. leucostictus* were still turning continuously in circles, but the diameter of the circles had increased approximately four times and the fishes swam at slower speed. Other reactions concerned with feeding and avoidance of obstacles were superimposed, to a greater extent than at the beginning, upon the circling reactions. When excited, however, the fishes both relapsed into their earlier rapid and intense circling behavior. One of the cases was found dead on the 25th day. The other continued to improve by swimming in circles of ever increasing diameter until it was traveling in ovals as large as the aquarium would permit. By the 32d day after operation it was able to swim straight forward most of the time. The final observations on this case were made on the 45th day after operation. At this time the fish ordinarily swam straight, but, when excited by the introduction of a stick or a net into the aquarium, it reverted to its typical forced circling activity. Its optokinetic reactions in the rotating drum were still made in the direction opposite from normal. Localizing responses to food objects and an approaching net, likewise, were still definitely in reverse. All the eight *B. soporator* survived in good condition for the full 45 days. No signs of any corrective re-education were noted in this species.

The present experiments were not designed to determine whether fishes can eventually adapt themselves to inversion of the retinal field. The tests were not administered frequently enough to serve as a basis for training, nor was any special effort made to train the fishes (for example, to avoid an approaching net). The results suggest, however, that adap-

tation is possible to some extent in certain species. The forced circling was corrected by two animals. Although no evidence of correction of the directional reversal of localizing responses was detected, certainly the possibilities of such adjustment with further training appeared to be much more promising in these fishes than in the amphibians.

The observations were carried far enough to determine the outstanding features of inverted vision, to show that it is highly maladaptive and that it is corrigible, if at all, only after a considerable period of time. The results demonstrate satisfactorily for the purposes of the experiment that eye rotation can be employed in fishes as an adequate means for determining whether learning or related types of functional adaptation play an essential role in the recovery of vision following optic-nerve regeneration.

SECTION OF THE OPTIC NERVE

Both optic nerves were transected by the method described above in nine *B. soporator*, eight *Thalassoma bifasciatum* (Bloch), five *Halichoeres bivittatus* (Bloch), four *A. saxatilis*, and one *A. analagous* (Gill). The fishes of this group ranged from approximately 2 to 8 cm. in length. In colliding with the aquarium walls and other objects, in failure to locate food quickly or to startle at sudden movements of objects around the aquarium or to escape a small net, and in various additional species-specific activities, the behavior of all cases was indicative of total blindness.

Restoration of vision tended to occur sooner in the smaller fishes. Startle responses and optokinetic reactions reappeared as early as the 6th and 7th days after operation in the smallest *B. soporator* and *H. bivittata*, respectively. By the 14th day after operation, the majority of fishes had recovered vision in at least one eye. By the 20th day all had recovered except the *A. analagous*. The latter began to show signs of recovery on the 26th day after operation and appeared to be well recovered on the 38th day. Two additional *A. analagous* had died on the 16th and 19th days, respectively, without having shown any signs of recovery.

After optic-nerve regeneration, visuomotor co-ordination appeared to be entirely normal in quality in all cases. The fishes turned in the correct direction in making the optokinetic reactions and in the escape and feeding responses. They were able to locate and swim directly to small particles of food sinking in the aquarium or lying on the bottom, to pursue efficiently other fish, to dart into small distant crevices when startled, and to avoid the aquarium fixtures and other obstacles when swimming at high speed. As far as the tests could determine, their behavior was indistinguishable from that of normal fish. No attempt was made to measure visual acuity, and this quantitative aspect of vision may well have been subnormal, owing to loss of fibers in the scar region and the failure of others to establish functional relations in the centers. The tests were sufficient, however, to show clearly that the re-establishment of functional relations between retinal field and brain centers had taken place in an orderly, discriminative manner. Systematic selectivity of some kind was clearly present in the recovery process.

OPTIC-NERVE SECTION WITH EYE ROTATION

Eye rotation was combined with severance of the optic nerve to find out whether the orderliness of functional recovery observed to follow optic-nerve regeneration in the preceding group was dependent upon any type of functional adaptation, such as learning or conditioning. If under these conditions the

fishes recovered vision which was systematically reversed like that produced by rotation of the eye with optic nerve intact, the orderliness of recovery must be ascribed to organizational factors operating in the regeneration process itself, independent of functional adaptation. In fourteen *B. soporator*, ranging in length from 21 to 63 mm., the eye on one side was rotated on its optic axis through 180°, and its optic nerve was severed. Twelve days after the primary operation the contralateral eye was excised.

On the 12th postoperative day, six of the fourteen cases already exhibited definite forced circling reactions following removal of the unrotated eye. By the 17th postoperative day, all fourteen cases were displaying the typical forced circling reactions indicative of reversed vision. When tested on the 18th day, all exhibited optokinetic responses in the direction opposite from normal. Elicitation of reversed localization and escape responses similar to those observed after simple eye rotation left no further doubt that the functional relationships between retina and brain had been restored in the same systematic manner in this group as in the preceding group. The fact that the recovery of function in this group was maladaptive, owing to the rotation of the eyes, ruled out functional adaptation as a possible factor responsible for the orderly adjustment of central synapses.

The fishes were all in the light-color phase when the unrotated eye was excised, including those that had already recovered vision. Consistent with the results of eye rotation with nerve intact, they all changed during the next several days into the dark phase, in which they remained until the termination of the experiments.

At the time of sacrifice, 41 days after the initial operation, these *B. soporator* were still exhibiting spontaneous circling reactions, reversed optokinetic reactions, and reversal of the localizing responses involved in feeding and flight.

HISTOLOGICAL CHECKS

Microscopic examination of the regenerated optic nerves following silver impregnation revealed that the nerve fibers had undergone copious regeneration from the peripheral stump and had re-established a large optic-nerve connection from the retina to the brain at the optic chiasma. The regenerated fibers were easily traced from the chiasma along their normal course into the contralateral optic lobe. In most cases the size of the optic nerve was not noticeably reduced beyond the point where it had been sectioned. Throughout the scar region the fibers were profusely interwoven with one another in fascicles of varying size (see Fig. 1). It was obvious that the orderliness of functional recovery had not been due to an indifferent mechanical guidance of fibers across the lesion into their original specific channels.

DISCUSSION

With respect to the manner in which the optic fibers re-established their central associations in the brain, the present findings are essentially the same as those obtained earlier in amphibians (Sperry, 1942–44). Previous discussions are thus applicable in large measure to the present results and need not be repeated. Despite haphazard intermixing of fibers in the scar region, the formation of synaptic relations took place in a systematic, strictly predetermined manner, with no regard for functional adaptiveness. The results indicate that the optic fibers in teleosts, as in amphibians, are qualitatively specified through embryonic differentiation of the retinal field and that this specification determines the types of synaptic associations formed in the centers. The re-

FIG. 1.—Photomicrographs of 10-μ sections of regenerated optic nerves of *B. soporator*. Optic fibers emerging from eyeball toward right run a tangled, tortuous course through the neuromatous scar before entering cental nerve stump at left. In *B* fibers running through central core of scar take a relatively direct route, but in *A* the fiber pattern, even in center of scar, is disorganized. *B* and *C* are from the same nerve, with *B* nearer center of scar. $\times 150$.

Despite this extreme disorientation of the regenerating optic fibers, the re-establishment of functional relations in the brain was orderly and systematic.

sults strengthen the supposition that the principles of synaptic formation derived from the amphibian studies are not peculiar to the class Amphibia but apply also to the nervous system of other vertebrates.

Visual recovery following optic-nerve division was slightly quicker in some of these fish than even the most rapid recovery obtained previously under similar conditions in amphibians (Sperry, 1944). Both function and histological appearance also indicated that regeneration in general was fully as good or better in the fishes. Failure of the optic nerve to reestablish central connections under similar conditions in *Fundulus heteroclitus* (Matthews, 1933) remains unexplained.

Correlated with their more highly developed optic brain centers, certain of the fishes showed greater promise than had any amphibians of being able to achieve some motor adaptation to the inversion of the retinal field. The beginnings of certain minor adjustments appeared in three free-swimming species. Two cases succeeded within the brief time-span of the experiments in correcting the forced circling reactions except under conditions of unusual excitement. Whether this represented an act of learning or of some other form of functional adaptation cannot be said without further analysis. The reversed localization of objects remained uncorrected in these cases. In contrast to the foregoing free-swimming species, the *B. soporator*, which cling to the bottom most of the time, failed to show any signs of re-education, although all eight of these animals survived in good condition for the entire observation period of 45 days.

SUMMARY

1. Rotation of the eyeball on its optic axis through 180° (with optic nerve and major blood vessels remaining intact) in fifteen fishes, including six species from four different families, produced in all cases definite symptoms of inverted vision, such as forced circling, reversed optokinetic reactions, and misdirected localizing responses. For the most part these reactions remained uncorrected within the 45-day observation period. The forced circling, however, after the first 2 weeks, began to be inhibited in varying degree in several of the free-swimming species.

2. Both optic nerves were divided, producing total blindness in twenty-seven fishes, including five species from three different families. All cases exhibited a rapid and excellent recovery of visuomotor reactions, which in character were indistinguishable from normal.

3. Optic-nerve division was combined with eye rotation on one side in fourteen *B. soporator*. The contralateral eye was excised. All cases recovered the systematically inverted type of visuomotor reactions such as result from eye rotation with the optic-nerve connections left intact.

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RESPONSES OF THE CHROMATOPHORES OF THE FIDDLER CRAB, UCA, TO LIGHT AND TEMPERATURE

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THE chromatophore systems of numerous animals are known to be significantly influenced by light and temperature. Although the specific quantitative relationships of these influences for vertebrates have received considerable attention, little is known at present concerning them for invertebrates.

Responses to light have been divided into two general types: (1) primary responses, which are responses to total illumination influencing the chromatophores either directly or reflexly, and (2) secondary responses, which are specific responses to background and are mediated exclusively through the eyes. The primary response is observed in many young vertebrates prior to the normal development of secondary control and also often following removal of the eyes in adults. The general character of these primary responses is, typically, dispersion of both black and white pigments as

total illumination increases. In secondary responses a concentration of dark pigment occurs when the animal is upon an illuminated white background, and dispersion occurs when it is upon an illuminated black background. White pigment usually reacts in an opposite fashion. The adaptive responses to backgrounds observed in a number of vertebrate and invertebrate species are of this character. It would appear that primary responses are the more primitive.

In the crustaceans the chromatophores receive no direct innervation; their secondary responses are therefore determined exclusively by hormones. These hormones are known to be secreted by the sinus gland of the eyestalk and by elements within the central nervous system.

Crustaceans in which primary responses as well as secondary have been demonstrated include species in the following genera: *Hippolyte* (Keeble and Gamble, 1900; Kleinholz and Welsh, 1937), *Eupagurus* (Stephenson, 1932), *Macromysis* and *Palaemon* (Keeble and Gamble, 1904), *Leander* (Stephenson, 1934), and *Ligia* (H. Smith, 1938).

In 1900 Keeble and Gamble postulated that, when animals adapted to the shade of the background, it was a response to the ratio of incident light to reflected light. Thus on a black background, where the ratio is relatively large, the animal becomes dark, while on a white background, where the ratio is relatively small, the animal becomes pale. This has also been found true for fishes (Sumner and Keyes, 1929; Pearson, 1930; Brown, 1936). Butcher (1938), working on *Fundulus*, demonstrated that this change in color was not simply a response to contrast in the visual field but rather a result of differentiation in the retina or in the nervous system associated with the retina. Although Keeble and Gamble (1904) could not localize retinal elements in *Hippolyte* by inversion of background and light source, H. Smith (1938) demonstrated such a localization in *Ligia* by opaquing various areas of the retina. He found that stimulation of the dorsal half of the eye produced a darkening of the animal, while stimulation of the lateral and ventral areas of the eye produced a blanching of the animal.

In *Ericymba* the ratio of incident to reflected light appears to determine totally the observed response when the illumination is higher than about 2 foot-candles. Below this value, total illumination appears to determine the response (Brown, 1936).

The primary responses in vertebrates have been demonstrated to be the result of the chromatophores acting as "independent effectors" in such species as *Xenopus* (Hogben and Slome, 1931) and *Phrynosoma* (Parker, 1938) and the result of reflex action in others, such as *Lampetra* larvae, in which the pineal body is involved (Young, 1935).

The responses to temperature are not so uniform as are the responses to light. Most vertebrates show a concentration of melanin within their melanophores at high temperatures and a dispersion at low temperatures (Parker, 1938). In crustaceans, concentration of dark pigments above 32° C. and below 8° C. has been reported for *Hippolyte* (Gamble and Keeble, 1900), and dispersion at 35°–40° C. and 6°–15° C. for *Macrobrachium* (D. C. Smith, 1930).

Most of the observations on responses to temperature seem to suggest that the chromatophores are acting as "independent effectors." This has been shown to be true for *Phrynosoma* (Parker, 1938). D. C. Smith (1930) made assays of sinus-gland activity of *Macrobrachium* kept at 15° C., 37° C., and room temperature and found all quantitatively equal. He de-

cided, therefore, that the response of the chromatophores to temperature, which he noted, was a direct effect.

Uca pugilator, which was utilized in the experiments to be described, possesses a 24-hour rhythm of color change, which is manifested by a pale phase at night and a dark phase during the daytime (Abramowitz, 1937*a*). The pale phase is a result of the concentration of both black and white pigments, while the dark phase is a result of the dispersion of both pigments. It has been stated that this periodicity is the only factor which determines color changes in *Uca* and that background adaptation is lacking (Abramowitz, 1937*a*).

The diurnal rhythm of color change in *Uca* has been reported to be abolished by the amputation of both eyestalks at their bases. This operation results in a concentration of black pigment and a dispersion of white, with these pigments no longer responding to light or darkness (Carlson, 1935, 1936; Abramowitz, 1937*a*, *b*). The rhythmic color change of *Uca* has been attributed to a rhythmical release of a chromatophorotropin secreted by the sinus glands of the eyestalks (Abramowitz, 1937*a*), with black-pigment dispersion resulting from the presence in the blood of a sinus-gland principle and concentration resulting from its absence. This explanation was offered in the belief that the sinus glands were the only sources of chromatophorotropin in *Uca*. It has since been demonstrated that extracts of the central nervous system contain a black-pigment-dispersing principle and principles which influence the state of the white pigment (Brown, 1948).

Some evidence indicating a continuance of a diurnally rhythmic production of chromatophorotropin in eyestalkless *Uca* was advanced by Brown and Scudamore (1940). They noticed that weak extracts of sinus gland were effective in producing black-pigment dispersal in the daytime but had little or no effect on the same or other animals at night. Thus it appears that the primary control of the diurnal rhythm lies in some tissue outside the eyestalk and that the observed changes in pigment dispersal are a result of responses to hormones produced both in the sinus gland and in the central nervous system.

The following study was conducted to determine whether the normal color changes of *U. pugilator* are influenced by environmental conditions, such as background, light intensity, and temperature, and, if so, to determine the relative influences of these factors in the normally observed states of the chromatophore system. There has been no previous attempt to analyze the relative influences of the various internal and external factors known to exert an effect upon the state of the chromatophore system in any single invertebrate. It was anticipated, furthermore, that a more precise knowledge of the normal characteristics of response might give some suggestion as to the specific functional significances of this system in *Uca*.

MATERIAL AND METHODS

Five groups of ten animals each, chosen at random from the stock supply, were placed in containers at a series of illuminations which included the following values (determined by a Weston Photometer): 300, 110, 50, 20, and 1 foot-candles. This gradient was obtained by using a 300-watt Mazda lamp in an otherwise darkened room. The desired illuminations were achieved by placing the containers with the animals at various distances from the light source.

Each experiment was continued for 5 consecutive hours, during which time each group of animals was exposed to each light intensity for 1 hour. At the end

of each hour the states of the black and white chromatophores of the animals were recorded, with the chromatophore index originated by Hogben and Slome (1931), in which 1 signifies maximum concentration of pigment, 5 maximum dispersion, and 2, 3, and 4 intermediate conditions.

The chromatophore index was determined by observing a leg of the animal with the aid of a dissecting microscope. The single value which was considered to approximate most closely the average condition of all the chromatophores in the selected area was recorded as the index for that animal. The chromatophores which were used consistently for the readings were those located on the anteroventral aspect of the proximal segment of the third or fourth walking leg. These chromatophores are in such a position that they are illuminated almost exclusively by the light reflected from the background.

The experiment was performed, first, by using a set of black containers and later by using a set of white ones. The white containers were white enameled pans; the black ones were aluminum pans coated internally with black enamel. The diameter of the pan bottoms was 9 inches. About 15–20 mm. of sea water, a depth sufficient partially to cover the animals, was placed in each container. The temperature of the water during the experiments varied between 23° and 25° C.

Experiments were conducted for each background during both the day phase and the night phase of the diurnal rhythm of color change. No observations were made on the state of the chromatophores during the night phase on a black background at 300 foot-candles.

To investigate the influences of temperature, two groups of ten normal animals each, one group in the black container and one in the white, were exposed to various temperatures in the following manner. Sea water to a depth of about 20 mm. was added to each container, so that the animals were nearly submerged. The containers were then placed in water baths. The temperature of the baths was regulated to provide various temperatures, ranging from 10° to 35° C. at 5° intervals. The variation at each temperature was not greater than 1° C. An incandescent light source, providing about 100 foot-candle illumination, was used for each group.

The experiment was conducted during the day phase of the diurnal cycle for several consecutive hours. Each group of animals was subjected to one temperature for 1 hour, the state of the chromatophores was recorded, and the temperature was adjusted to the next higher value. It usually required about 10 minutes for the new temperature to be established.

RESULTS AND CONCLUSIONS

LIGHT

The average indices for the black and white chromatophores for each group of ten animals were determined, and then an over-all average was calculated for the five groups for each intensity of light. In Figures 1 and 2 the average chromatophore index for fifty animals at each intensity of light is plotted against the logarithm of the incident light intensity.

It seemed desirable to obtain a much higher light intensity in order to determine whether the apparent tendency toward a convergence of curves *A* and *B* in Figures 1 and 2 was real. To do this, a group of ten animals in a black container and another group of ten animals in a white one were exposed to sunlight at noon on a bright day. The illumination was measured and found to be approximately 3,500 foot-candles. The average

chromatophore index of each group is included in Figures 1 and 2.

Another experiment was conducted with animals whose eyestalks had been removed 2–3 days previously. As a result of this operation the black pigment was concentrated and the white dispersed. These animals were exposed to the light gradient in the same manner as described

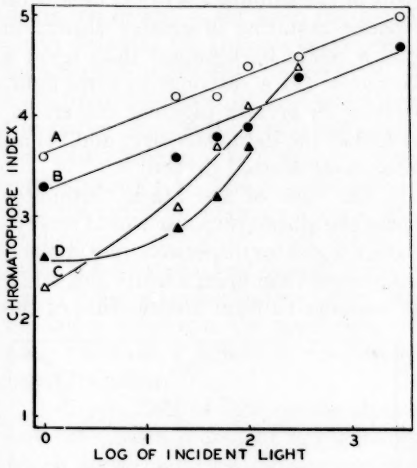

FIG. 1.—The relationships between the black-chromatophore indices of *Uca* and the logarithm of the light intensity for animals in the day phase on a white background (*A*), day phase on a black background (*B*), night phase on a white background (*C*), and night phase on a black background (*D*).

previously, with a white background in the daytime. Only two trials of ten animals each were conducted for the four lower light intensities, inasmuch as these intensities appeared relatively ineffective; but five trials of ten animals each were made for the 300 foot-candle illumination. The results of this experiment are shown in Figure 3.

In order to assist in the analysis of the foregoing data, Figure 4 was prepared. In this figure the chromatophore index is plotted against the logarithm of the reflected illumination. By means of a Weston Photometer it was found that the white background diffusely reflected approximately 40 per cent of the incident light. Furthermore, the white background was found to reflect about fifty times as much light as was reflected from the black background for any given incident illumination.

From Figures 1 and 2 a number of facts are evident concerning both the black and the white chromatophores.

For any given illumination the ordinate distances between curves *A* and *C*, on the one hand, and curves *B* and *D*, on the other, indicate the influence of an endogenous diurnal rhythm. In the day

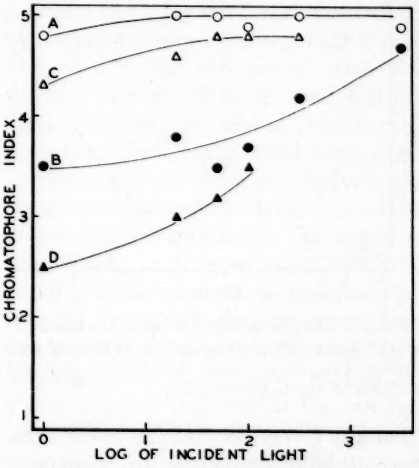

FIG. 2.—The relationships between the white-chromatophore indices of *Uca* and the logarithm of the light intensity for animals in the day phase on a white background (*A*), day phase on a black background (*B*), night phase on a white background (*C*), and night phase on a black background (*D*).

phase of the diurnal cycle the animals show a greater dispersion of both pigments for any given light intensity than they do in the night phase of their rhythm.

There is in every instance an increase in pigment dispersion with an increase in total illumination. If we consider each

curve individually, the animals are in the same phase of their diurnal cycle, and the background is constant. Since the only factor which is varying here is total illumination, the chromatophores, both black and white, are definitely responding to total illumination, since increasing illumination tends to produce greater degrees of pigment dispersal.

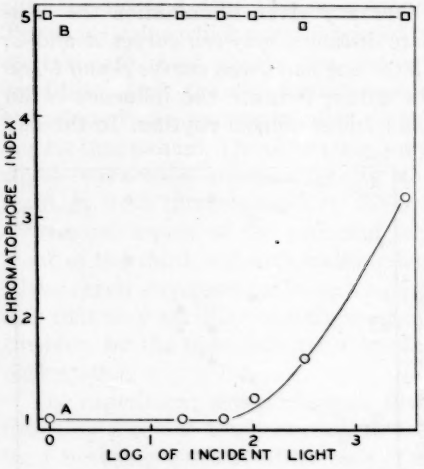

FIG. 3.—The relationships between the black (*A*) and the white (*B*) chromatophore indices of eyestalkless *Uca* on a white background and the logarithm of the light intensity.

For any given illumination in the same phase of the diurnal cycle, the dispersion of the white pigment is greater upon a white background than upon a black background. This might at first be considered due to the differences in the background-reflected light impinging upon the chromatophores in question, which are located upon the ventral surfaces of the legs, rather than to a specific albedo response (response to ratio of incident to reflected light) mediated by the eyes. That an albedo response is operating here, however, is evident from the fact that there is no overlapping of any values upon the black and white backgrounds for any given phase of the diurnal cycle, despite a three hundred fold or more increase in incident illumination, as compared with only a fifty fold increase for the white over the black background reflection.

It is apparent by inspection that the responses of the white chromatophores shown in the curves in Figure 2 are the result of the summation of (1) an albedo response, resulting in greater dispersion upon a white background than upon a black one; (2) a response to total light, resulting in greater pigment dispersion, the higher the light intensity; and (3) an endogenous diurnal rhythm.

In the case of the black chromatophores the albedo response would tend to produce a greater dispersion upon a black background than upon a white one, while the response to total illumination of the

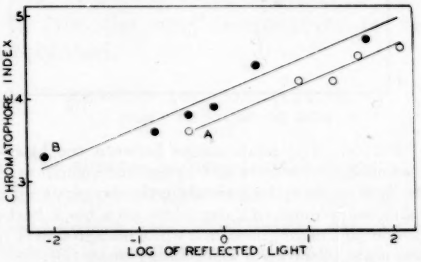

FIG. 4.—The relationships between black chromatophores and the logarithm of the reflected light in *Uca* in the day phase kept on a white background (*A*) and on a black background (*B*).

melanophores results in a greater dispersion upon a white background than upon a black background. Therefore, the albedo response of these chromatophores is antagonistic to the response determined by the background acting as a modifier of total illumination.

By inspection of Figure 4 it seems evident that an albedo response is operating for the black chromatophores. Thus, for any given value of reflected light, the dispersion of pigment is greater upon a

black background than upon a white one. And that total illumination is operating simultaneously is confirmed because there is a greater dispersion, the higher the reflected light intensity. The fact that the difference between the two curves is so small may raise the question of the significance of the albedo response. However, in no case is the amount of deviation of any point from the plotted curve obtained for animals upon a white background sufficient to overlap the maximum deviation of any point from the curve obtained for animals upon a black background. The difference, therefore, appears real. The average chromatophore index for animals upon a white background at 3,500 foot-candle illumination (1,400 foot-candle reflected light) is not included in this figure, since the curve intercepts the maximum ordinate value of 5 before it reaches this intensity value.

A direct effect of light on the chromatophore system is evident in Figure 3, in which all secondary responses are eliminated by the removal of the eyestalks. The apparent absence of an influence of total illumination upon the white chromatophores and, more obviously, upon the black at the lower illuminations in all probability owes its explanation to the removal of the sinus glands along with the eyestalks. Under these circumstances it would appear that the balance of internal humoral factors maintains these chromatophores quite rigidly in the extreme states. Only at high values of illumination, in the case of the black pigment, are the influences of illumination sufficient to overcome these internal governing forces.

It is evident from the results of these experiments that changes in the chromatophore state of *U. pugilator* can occur in response to amount of illumination, although the diurnal rhythm of color change is perhaps a more effective influence. A specific background or albedo response (secondary response) and a response to total illumination (primary response) have been demonstrated.

Although eyestalkless animals show a primary response of the black pigment to light intensity at illumination values above 50 foot-candles, this does not distinguish between the possibilities that in normal animals (1) the chromatophores are responding directly to the light and (2) the response is reflex, operating by way of photoreceptors, including the eyes.

It has been reported that the primary response has a higher threshold than the secondary response has and that it is active only in the absence of other controlling factors (Keeble and Gamble, 1904; Hogben and Slome, 1931). Since both these conditions could exist, it has not been demonstrated conclusively that the curve obtained in Figure 3 is a result of this higher threshold. The curve for the black chromatophores could be equally well explained, as described earlier, in terms of the chromatophores being held in a concentrated condition by some humoral mechanism. In this case the absence of response at the low light intensities could be the result of antagonism by a concentrating hormone.

In view of these various possible explanations for this primary effect and since the influence of total light on normal animals is evident throughout the gradient of light intensities used, these data do not permit the identification of the mechanism of response to total illumination.

Similarly, the specific mechanism of the albedo response awaits further elucidation. The animals may be responding simply to general contrast in the visual field, or physiologically differentiated and localized areas in the retina may be operating.

TEMPERATURE

The average chromatophore index for the ten animals at each temperature was obtained. In the first experiment it was found that 40° C. was lethal for three out of the ten animals, so no further experimentation was done at this temperature.

Readings were not taken in this first test trial at 20° and 25° C. The values for the 25° C. temperature were obtained from the 110 foot-candle averages used in Figures 1 and 2, which, as has been previously stated, were obtained from fifty animals at a temperature of 23°–25° C. The difference of 10 per cent in illumination is not important, since it has been found to require a much greater change to produce a measurable difference in chromatophore index.

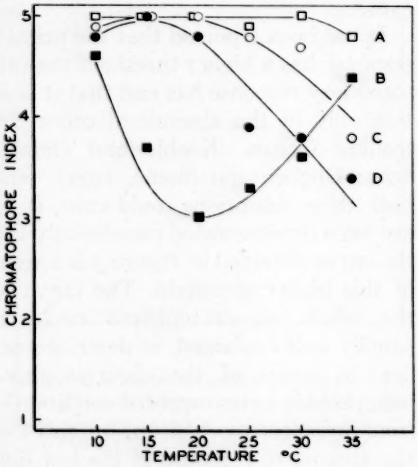

FIG. 5.—The relationships between the black- and white-chromatophore indices and temperature in *Uca* during the day phase of the diurnal cycle. *A* represents white chromatophores on a white background; *B*, white chromatophores on a black background; *C*, black chromatophores on a white background; and *D*, black chromatophores on a black background.

In the second trial the two groups of animals were exposed to the six different temperatures in succession, and the same trend as that observed in the first trial was apparent. The average chromatophore index for the ten animals was obtained. The values for each temperature in these two experiments were then averaged and plotted against temperature in Figure 5.

An examination of Figure 5 reveals the following relationships between temperature and chromatophore index.

There are differences in response between animals upon a black background and animals upon a white one. In the case of the black chromatophores this is a difference in degree, while in the case of the white chromatophores it is a difference in kind of response.

Upon both backgrounds the black pigment tends to concentrate as the temperature increases above 15° C.; the higher the temperature, the greater is the degree of concentration. This tendency is greater upon a black background than upon a white. There is also suggested an apparent tendency of the black pigment to concentrate at temperatures lower than 15° C., although in this experiment only one more value, 10° C., was used.

Upon a white background the white pigment showed a tendency to concentrate only when the temperature reached 35° C., while on a black background it exhibited its maximal tendency to concentrate at a temperature of about 20° C. and to disperse more and more as the temperature was gradually increased or decreased from this value.

Albedo response is, therefore, of maximum relative influence at about 20° C. for the white and at about 15° C. for the black pigment.

It seems apparent from these observations that, although the chromatophores of *Uca* respond to changes in temperature, these responses are superimposed upon an existing response to light, since an influence of both background and to-

tal illumination is evident. At all temperatures there is usually greater, never less, dispersion of pigment upon a white background than upon a black background.

The response of the black pigment to temperature in *Uca* is, therefore, like that reported for *Hippolyte* by Gamble and Keeble (1900) and the opposite to that reported for *Macrobrachium* by D. C. Smith (1930).

Since removal of the eyestalks produces dispersion of pigments in *Macrobrachium* and the response to high and low temperatures results in dispersion, D. C. Smith (1930) was not able to demonstrate a direct effect upon the chromatophores by exposing eyestalkless animals to different temperatures.

Uca sinus gland contains a principle which induces dispersion of its black pigment (Carlson, 1935). *Hippolyte* sinus gland contains a hormone which induces concentration of its dark pigment (Kleinholz and Welsh, 1937). Despite this difference, the response to high and low temperatures is the same in both these species. Therefore, if the effect were directly upon the sinus gland, one would need to postulate that the influence of temperature was the opposite in these two instances.

It is evident from Figure 5 that the response of the black pigment of *U. pugilator* between the temperatures of 15° and 20° C. is opposite to that reported for *U. pugnax* by Megušar (1912), who reported that the dark pigment concentrated when the animals were suddenly transferred from a higher temperature to 16°–18° C. This difference is probably accounted for by the differences in procedure employed. The animals in the present experiment were allowed to equilibrate at the various temperatures for an hour before the state of the chromatophores was determined.

GENERAL DISCUSSION

Since the diurnal rhythm of *U. pugilator* is such a strong factor in determining the color of the animals, the weaker albedo response does not provide any substantial background adaptation. A background response of the magnitude observed probably could not serve in producing obliterative coloration of survival value.

On the other hand, the dispersion of pigments in response to high intensities of illumination may result in a protection of the protoplasm of the animal. In the night phase of the diurnal cycle, when both pigments are concentrated, the legs of the animal are relatively transparent, in contrast to the day phase, in which pigment dispersal renders the integument quite opaque. In the daytime, therefore, there is a region preventing transmission of light to deeper tissue areas. The observed responses of both black and white pigments to total illumination appear adaptive ones in these terms.

The evidence presented here favors postulating a thermoregulatory role for the chromatophores of *Uca*. Optimal conditions of the chromatophore system to produce dissipation of light when the temperature of the environment is high would be, as is the actual case, a dispersion of white pigment and a concentration of the black. The concentration of the black tends to diminish the area which absorbs the light, while the dispersion of the white tends to increase the area which reflects it.

Recently Deanin and Steggerda (1948) demonstrated spectrophotometrically that more light is reflected from the skin of a frog adapted to a white background (black pigment concentrated) than from a frog adapted to a black background (black pigment dispersed). These authors have shown that there is greater reflec-

tion of the wave lengths at the red end of the spectrum than at the violet end. This seems significant in view of the greater heating capacity of the red rays than of the violet rays, which thus offers additional support for an effective thermoregulatory function of color change.

Only at the intermediate temperature range, roughly 15°–25° C., is background response relatively important in determining the chromatophore state; beyond this narrow temperature range, adaptive responses to total illumination and temperature appear to predominate.

SUMMARY

The condition of the black and white chromatophores of *U. pugilator* is determined by several factors operating simultaneously as follows:

1. There is an endogenous rhythm of chromatophorotropin secretion, which operates to disperse the black and white pigments in the day phase of the cycle and to concentrate the black and white pigments in the night phase of the cycle.

2. There is a specific background, or albedo, response which operates to concentrate black pigment and disperse white pigment upon a white background and to disperse black pigment and concentrate white pigment upon a black background.

3. There is a response to total illumination which results in an increased dispersion of both black and white pigments as the intensity of illumination increases.

4. There is a response to temperature, with the black pigment tending to concentrate as the temperature is increased above, or decreased below, about 15° C. The white pigment, on the other hand, tends to disperse as the temperature is increased above, or decreased below, about 20° C.

A possible adaptive significance of these chromatophore responses in *Uca* is discussed.

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TEMPERATURE RELATIONS OF AN ENDOGENOUS DAILY RHYTHMICITY IN THE FIDDLER CRAB, UCA

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ONE of the most challenging problems in biology is presented by the phenomenon of rhythmicity, exhibited by a wide variety of animals and plants. Although many valuable contributions have been made in work on this general problem, many of the experiments have been devised to isolate factors other than those responsible for the phenomenon itself. It has been found that numerous processes which give evidence of cyclic activity continue in a rhythmical manner under constant conditions of light, temperature, and humidity—conditions which normally influence the response of the effectors involved. Thus a persistent diurnal rhythm of retinal-pigment migration in the eyes of a noctuid moth, *Plusia gamma*, was reported by Kiesel as early as 1894. Welsh (1930) demonstrated a diurnal pigment migration in the eyes of the freshwater shrimp, *Macrobrachium*, which persisted under constant illumination. Bennitt (1932) extended these observations to include the crayfish, *Cambarus*. Park and Keller (1932) reported that the beetle, *Boletotherus cornutus*, showed a continued periodic activity under constant conditions of darkness, temperature, and evaporation rate. Such periodicity was evident even in animals that were raised from the larval stage in the laboratory under conditions of constant darkness.

Welsh (1941) stated that the diurnal retinal-pigment migration of *Cambarus* persisted at the same frequency in animals maintained in constant darkness and at constant temperatures. One group of animals was maintained at 7° C. and another at 21° C. The only reported difference between the two groups was the length of time for which the rhythms persisted, the time being somewhat shorter at the higher temperature.

Another example of diurnal rhythm is a periodic color change, such as that reported for the prawn, *Hippolyte varians*, by Keeble and Gamble (1900). These authors reported that the rhythm persisted under constant conditions of illumination. Although the persistence of the diurnal color change in *Hippolyte* has been unconfirmed by subsequent investigators (Kleinholz and Welsh, 1937), a

similar condition has been established in many other animals, including the walking-stick insect, *Dixippus* (Schleip, 1910), and an isopod, *Idothea* (Menke, 1911), as well as in many species of vertebrates. In 1937 Abramowitz reported a persistent diurnal color change in the fiddler crab, *Uca*.

The question of the mechanism of control has been discussed most frequently in terms of the specific process under investigation. The explanations which have been offered for the phenomenon of persistent diurnal rhythms may be divided into two general groups: those involving environmental forces external to the animal and those involving endogenous forces. The former would include rhythmically varying physical factors, such as light rays (within and without the visible spectrum), temperature, humidity, and possibly other forces. Three of these factors (visible light, temperature, and humidity) are known normally to influence the responses of animals either by evoking a specific direct response or by modifying the response to simultaneous stimuli. They are all, however, relatively easy to control in the laboratory and have been ruled out as primary factors, since diurnal rhythms are generally referred to as "persisting" only when these factors are held constant.

The endogenous mechanisms suggested as explanations for persisting periodicity have been, in general, such as would explain the observed expressions of the rhythm (e.g., the eyestalk hormone for crustacean pigment migration), but the basis of the rhythm itself remains unexplored. In this connection it seems of prime importance to consider those experiments in which the frequency of the rhythm is undisturbed by widely different temperatures. The influence of temperature on biological processes has been recognized for many years. Excluding purely physical and photochemical responses, the van't Hoff temperature coefficients for the numerous biological processes which have been investigated generally lie between 2 and 3, rarely less than 2.

If 24-hour rhythmicity is a biological phenomenon based upon metabolic processes and has a temperature coefficient of 1.0, it appears to be unique in possessing this latter characteristic. The condition is the more surprising, in that it very commonly appears in cold-blooded animals. Such animals have no mechanism for maintaining a constant body temperature independent of the environment, and therefore their processes would be expected to vary in rate with external temperatures. A cyclic pattern of metabolic activity, such as has been suggested as a basis of diurnal color changes, would be expected to have its frequency influenced by temperature, much as does, for example, the inherently rhythmical heart of vertebrates. Inasmuch as no temperature-compensating mechanism has ever been proved to exist in cold-blooded animals, the temperature of the environment might be assumed to be the determining factor in the frequency of the rhythm.

On this assumption the first series of experiments reported here were designed to test whether the temperature of the environment influenced to any significant degree the frequency of the periodic color change in *Uca* (Brown and Webb, 1947).

A phenomenon that has been extensively studied and that exhibits a persistent 24-hour rhythm is "time-memory" in bees. Beling (1929) reported that this process apparently operated within a cycle of 24 hours, since she was unable to train the animals to approach an artificial feeding station at 19-hour intervals. They could, however, be trained to come

at any hour of the day and even two or three times in a single day but tended to return at the same hours the day following. Grabensberger (1934) found that feeding quinine to trained bees resulted in delayed arrival at the feeding station, while iodothyroglobulin hastened the arrival. Kalmus (1934) demonstrated that very low temperatures (5°–7° C.) retarded the cycle but that within the range of 18°–32° C. there was no influence of temperature. On the basis of this work he postulated an internal mechanism of control of the rhythmicity and also a mechanism of temperature compensation effective within a limited range.

If there exists in *Uca* some rhythmical metabolic process and if the diurnal color change is an expression of such a cycle, then a temporary reduction of all metabolic processes to a minimal level might be expected to modify the cycle of color change. To test this possibility, three groups of animals were exposed to a temperature of about 0° C. for a period of 6 hours and immediately thereafter placed in constant darkness.

MATERIAL AND METHODS

The animals used in the following experiments were specimens of *Uca* collected near Woods Hole, Massachusetts. Those used in the first group of experiments were collected early in July and were kept in sea water in the laboratory. Those for the second group were collected in part in November and in part in May and shipped to Evanston, Illinois. They were kept in tanks in the laboratory with a small amount of sea water.

1. Three groups of ten animals each were used in each series of experiments. The animals were placed in wooden, light-proof containers (bottom diameter, 10 inches), each of which contained sea water almost sufficient to cover the animals. The water was changed every 3 days during the course of an experiment. One group of animals was maintained at about 26° C., the room temperature of the laboratory; a second group was kept at about 16° C. in a cold room at the Marine Biological Laboratory, where the temperature was constantly within a range of 15°–17° C. The third group was placed in another cold room at the Marine Biological Laboratory, where the stated temperature was 6° C. Although on rare occasions the temperature was found to be as high as 9° C., it was considered that this temperature did not occur frequently enough to be important. Thermometers were placed in all containers in such a way that the water temperature could be checked without disturbing the animals.

The states of dispersion of the black and the white pigment were determined at 7:00 A.M., 1:00 P.M., 7:00 P.M., and 1:00 A.M. and recorded, with the chromatophore index of Hogben and Slome (1931). In the use of this method the most concentrated condition of the pigment is described as "stage 1," the most dispersed as "stage 5," while intermediate conditions are referred to as "stages 2, 3, and 4"—representing increasing degrees of dispersion. The chromatophores on the anteroventral aspect of the leg were used. The average for the group was then determined.

In the first series of experiments, chromatophore indices were recorded four times daily, at the times indicated above, in the period from July 16 to July 22. A second series was set up under similar conditions on July 22, but no readings were taken until August 5. During this 2-week period the animals were undisturbed except for brief periods every 3 days, when the water in the containers was changed. The chromato-

phore index was determined for all animals five times in a 24-hour period, beginning at 1:00 P.M., August 5, and ending at 1:00 P.M., August 6. One week later, beginning at 7:00 A.M., August 12, another series of observations was made at 6-hour intervals, the last being at 1:00 P.M. August 13. A final series of observations was recorded, beginning at 7:00 A.M., August 19, and ending at 1:00 A.M., August 20.

2. One group of five animals was placed in a large Erlenmeyer flask with sufficient sea water to cover them, and

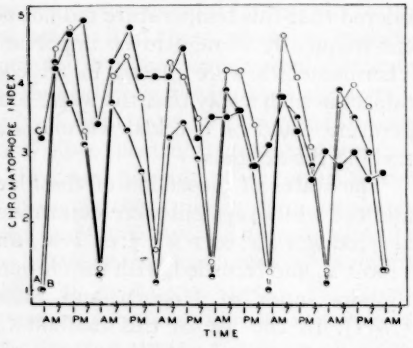

FIG. 1.—Daily variation in the average indices of black chromatophores in *Uca* kept in constant darkness at 26° C. (*A*), 16° C. (*B*), and 6° C. (*C*).

the flask, in turn, was immersed in a sea-water–ice mixture such that the sea water inside the flask was maintained between 0° and 3° C. for a period of 6 hours. The animals were then removed and placed in the wooden, light-proof containers which had been used in the first series of experiments. A control group of five animals was placed in a similar container at the same time, and both groups were kept in a room in which the temperature during the course of the experiment varied from 15° to 18° C. The chromatophore indices of the animals of both groups were determined at the times of day previously described.

In a repetition of this experiment, four groups of ten animals each were placed in a dark room (temperature 24°–25° C.), and the chromatophore indices were determined at 6-hour intervals for 3 days. At the end of this time two of the groups were placed in sea water in Erlenmeyer flasks and maintained at 0°–3° C. for 6 hours, as described for the group of five animals above. At the end of 6 hours the animals were returned to glass containers with a small amount of sea water, left in the dark room, and the chromatophore indices of these animals and of the controls were determined at the regular times for 6 more days.

RESULTS

Curve *A* in Figure 1 shows the characteristic rhythmic variation of the black chromatophores of *Uca*. The curve was obtained by plotting the observed chromatophore index against time. It can be seen that at 1:00 A.M. the chromatophores are at or near stage 1—the condition of maximal pigment concentration. At 7:00 A.M. a dispersion of the pigment within the chromatophores is evident, relative to the condition at 1:00 A.M. At 1:00 P.M. the pigment is also more or less dispersed, while at 7:00 P.M. it is always more concentrated than during the earlier hours of the day. Thus the night phase of the rhythm is characterized by a concentration of the black pigment and the day phase by a dispersed condition. The degree of dispersion or concentration of the pigment appears to be approximately the same on the sixth day (the last day recorded on this graph) as on the first day.

Curve *B* in Figure 1 represents the chromatophore indices of the animals maintained at 16° C. Here again the characteristics of the diurnal rhythm are clearly seen. This curve shows that these animals also were in the night phase,

with the chromatophores at or near stage 1, at 1:00 A.M., while the most dispersed stage is recorded either at 7:00 A.M. or at 1:00 P.M. It can be seen that the degree of dispersion of the pigment is consistently less in curve *B* than in curve *A*. There is no indication in curve *B* of any alteration in the frequency of the rhythm.

Curve *C* in this figure represents the average chromatophore indices of the animals maintained at approximately 6° C. Although this curve is obviously more irregular so far as the time at which maximal concentration of the pigment occurs, there is at no time any indication of a rhythm with a frequency different from that of the other two groups of animals. A complete cycle is obvious for any 24-hour period as long as the changes recorded can be considered significant. By the sixth day, when the chromatophores are consistently within the range of stages 2.5–3, the rhythm is no longer discernible.

Figure 2 represents the average chromatophore indices for the animals which were maintained under constant conditions for 4 weeks, during which time observations were made at the end of 2 weeks, again at 3 weeks, and, finally, after 4 weeks. The breaks in the graphs represent the times when observations were not made. Curve *A* is that of the animals maintained at 26° C. and shows clearly that at the end of 2, 3, and 4 weeks the diurnal rhythm retains its characteristic features. In any 24-hour period a cycle is completed and the most concentrated condition of the pigment is recorded at 1:00 A.M.

Curve *B* in Figure 2 shows the chromatophore indices of the group of animals maintained at 16° C. at the times when they were observed. There is no evidence that the frequency of the rhythm of these animals is different from that of the animals kept at 26° C. The greatest dispersion of the pigment is less than that seen in curve *A* of this figure, but there is little evidence of a continued decrease with time. The difference between curves *A* and *B* at points representing greatest dispersion of the pigment is about the same in the second week as in the fourth week and is approximately the same as that on the second day, as recorded in Figure 1.

Curve *C* of Figure 2 represents the average chromatophore indices of animals maintained at approximately 6° C. Since the change observed in these animals did not appear to be diurnally rhythmic after 3 weeks, these observations were not continued beyond that time. As can be seen, the variations recorded extended only from stage 2 to stage 2.5.

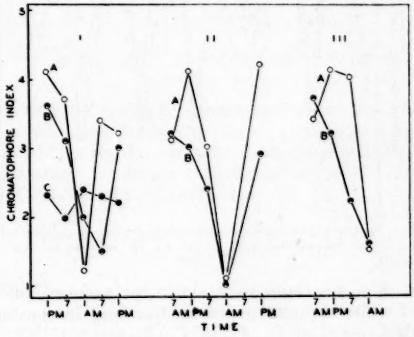

FIG. 2.—Daily variation in the average indices of black chromatophores in *Uca* kept in constant darkness for 2 weeks (*I*), 3 weeks (*II*), and 4 weeks (*III*) at 26° C. (*A*), 16° C. (*B*), and 6° C. (*C*).

Figure 3 is similar to Figure 1, except that now the average white chromatophore indices are represented. Curve *A* shows the condition of the white pigment of animals kept at 26° C., curve *B* at 16° C., and curve *C* at 6° C. It is obvious that the characteristics of these curves are very similar to those shown to prevail for the black chromatophores under

similar conditions. The most concentrated condition is recorded at 1:00 A.M., while at the other times when observations were made the pigment is considerably more dispersed. The rhythm is just as clearly evident on the sixth day as on the first at both 16° and 26° C. At 6° C. there are some irregularities in the rhythm, but it does seem possible to identify diurnally rhythmic changes on

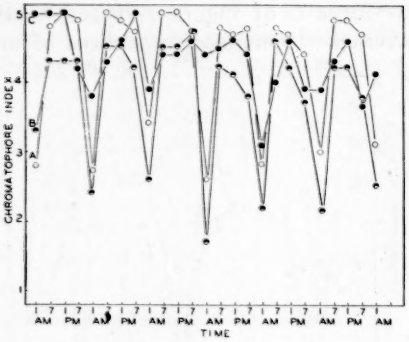

FIG. 3.—Daily variation in the average indices of white chromatophores in *Uca* kept in constant darkness at 26° C. (*A*), 16° C. (*B*), and 6° C. (*C*).

the sixth day. It will be recalled that this was not true of the black chromatophores.

Figure 4 is comparable to Figure 2, but here the average white chromatophore indices are represented. Curve *A* represents the condition of the white pigment at 26° C., curve *B* at 16° C., and curve *C* at 6° C. It is obvious that the diurnal rhythm is persisting to approximately the same degree throughout the course of the experiments at 16° and 26° C. The degree of concentration of the pigment in the night phase seems to be consistently less at 16° than at 26° C. The diurnal rhythm at 6° C. has ceased to be clearly observable after 2 weeks, and for this reason the observations at this temperature were discontinued.

Figure 5, *I*, *II*, and *III*, was obtained by plotting two curves for each group of animals, one representing the chromatophore index of the group during the night phase (1:00 A.M.) on each day on which observations were made, and the other curve representing the day phase (the average of the other three determinations). The data in this figure are for the black chromatophores. Any one curve, then, contains data from two series of animals, those represented in Figure 1 and those in Figure 2.

A comparison of *I*, *II*, and *III* of this figure shows very clearly the decreasing difference between night and day phases as the temperature is lowered. There appears also to be a tendency toward the convergence of the two curves representing data from groups of animals main-

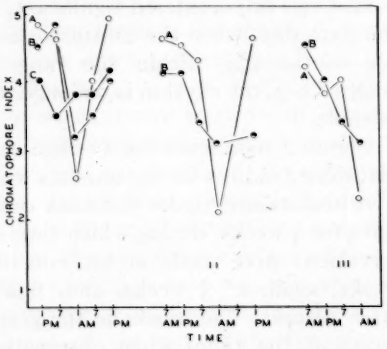

FIG. 4.—Daily variation in the average indices of white chromatophores in *Uca* kept in constant darkness for 2 weeks (*I*), 3 weeks (*II*), and 4 weeks (*III*) at 25° C. (*A*), 16° C. (*B*), and 6° C. (*C*).

tained at any one temperature, and this tendency is more marked at the lower temperatures. One very obvious difference between *III* of Figure 5 (animals kept at 6° C.) and *I* and *II* of the same figure is the continuously increasing concentration of the pigment shown in *III*. Thus the pigment is more dispersed in these animals in both the day and the night phases for the first few days and be-

comes more and more concentrated in both phases as time goes on. However, the day phase shows this effect more than does the night phase, as is shown by the definite convergence of the curves referred to above.

Figure 6 corresponds to Figure 5, but now the white chromatophores are represented. It can be seen that there is a slight but steady decrease in the degree of dispersion of the pigment in the day phase at all temperatures and in the

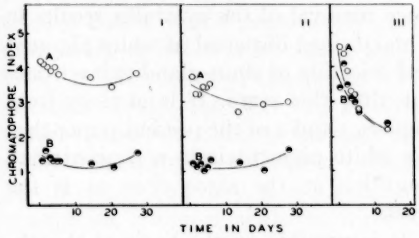

FIG. 5.—Changes in the average indices of black chromatophores in the day phase (*A*) and night phase (*B*) of the diurnal cycle of color change of *Uca* maintained in constant darkness at 26° C. (*I*), 16° C. (*II*), and 6° C. (*III*).

night phase at 26° C. throughout the experiment. A definite tendency toward an increase in the degree of dispersion is recorded for the night phase at 16° C. This latter results in an apparent convergence of the two curves at 16° C., indicating a smaller amount of daily change at the end of the experiment. The most outstanding feature of the curves at 6° C. is the marked and rapid convergence that they show.

Figure 7 illustrates the results obtained after maintenance of *Uca* at 0°–3° C. for 6 hours, followed by their immediate removal to constant darkness and a constant temperature of approximately 16° C. Curve *B* represents the chromatophore indices of a control group. It is immediately evident that both curves indicate the existence of a typical 24-hour rhythm. However, it is obvious now that curve *A* is consistently out of phase with curve *B*, and the extent to which it is out of phase appears to be about 6 hours. The experiment was repeated with the results summarized in Table 1. These results, it will be readily observed, completely confirm the earlier ones.

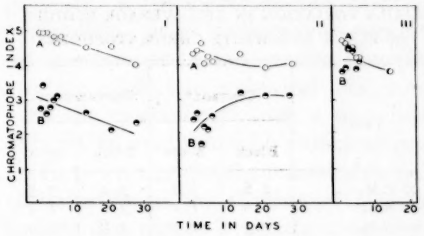

FIG. 6.—Changes in the average indices of white chromatophores in the day phase (*A*) and the night phase (*B*) of the diurnal cycle of color change of *Uca* maintained in constant darkness at 26° C. (*I*), 16° C. (*II*), and 6° C. (*III*).

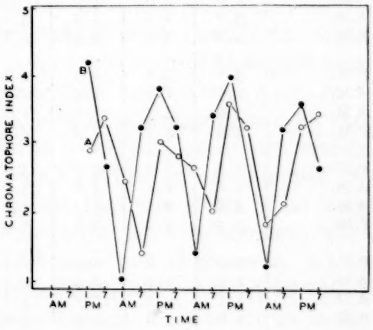

FIG. 7.—Daily variation in the average indices of black chromatophores in constant darkness in normal *Uca* (*B*) and in *Uca* which had been kept for 6 hours at 0°–3° C. just before the experiment began (*A*).

DISCUSSION AND CONCLUSIONS

As has been stated in describing the results obtained in these experiments, the diurnal rhythm shown by the white chromatophores is similar to that shown by the black. It is worth noting that previous observations of diurnal rhythm

in *Uca* have involved either the gross color changes undergone by the animal or detailed observations of the melanophores. So far as is known, no observations on the white chromatophores participating in the diurnal rhythm have been published.

TABLE 1

DAILY VARIATION IN THE AVERAGE INDICES OF BLACK AND WHITE CHROMATOPHORES

Time	Control*		Experimental†	
	Black	White	Black	White
7:00 P.M.	2.8	3.4	3.4	3.5
1:00 A.M.	1.0	1.1	1.0	1.5
7:00 A.M.	2.3	2.7	2.6	2.9
1:00 P.M.	3.5	3.4	4.0	3.6
7:00 P.M.	2.9	3.2	3.4	3.4
1:00 A.M.	1.1	1.3	1.0	1.5
7:00 A.M.	2.3	3.4	3.7	3.6
1:00 P.M.	4.1	3.9	4.1	3.8
7:00 P.M.	3.4	3.6	4.7	3.6
1:00 A.M.	1.0	1.3	1.0	1.5
7:00 A.M.	3.2	3.3	3.5	3.4
1:00 P.M.	3.2	3.4	3.9†	3.8†
7:00 P.M.	3.1	3.1	4.5	3.8
1:00 A.M.	1.1	1.3	2.9	2.6
7:00 A.M.	3.6	3.5	1.5	1.8
1:00 P.M.	3.9	3.7	4.2	4.1
7:00 P.M.	3.5	3.2	4.2	3.4
1:00 A.M.	1.1	1.1	2.8	3.2
7:00 A.M.	3.5	3.5	1.3	1.9
1:00 P.M.	3.5	3.2	2.6	2.4
7:00 P.M.	3.0	2.6	2.3	2.2
1:00 A.M.	1.2	1.4	2.9	2.7
7:00 A.M.	4.1	3.1	1.5	1.8
1:00 P.M.	4.6	3.6	4.5	3.8
7:00 P.M.	3.5	2.3	4.1	3.5
1:00 A.M.	1.1	1.2	3.2	2.8
7:00 A.M.	4.0	2.9	1.5	1.9
1:00 P.M.	4.1	3.3	4.4	3.7
7:00 P.M.	3.1	2.7	3.9	3.7
1:00 A.M.	1.1	1.9	2.3	3.1
7:00 A.M.	2.8	2.8	1.8	1.9
1:00 P.M.	3.2	3.5	4.4	4.0
7:00 P.M.	2.0	3.0	4.5	3.8
1:00 A.M.	1.3	1.6	2.6	3.1
7:00 A.M.	3.8	3.3	1.9	2.4
1:00 P.M.	4.4	3.8	4.9	4.0

* Normal *Uca* kept in constant darkness.

† *Uca*, in constant darkness, which were kept at 0°–3° C. for 6 hours at point in table indicated by †.

Abramowitz (1937) has suggested that the basis for the observed diurnal rhythm of *Uca* chromatophores lies in the presence of an eyestalk hormone in, or its absence from, the body fluid. The suggestion was based upon the well-known observation that removal of the eyestalks with their included sinus glands resulted in a permanent concentration of black pigment. Injection of eyestalk extract dispersed the pigment.

The white chromatophores are obviously controlled in another manner, since removal of the eyestalks results in a maintained dispersal of white pigment and injection of sinus-gland extract does not alter this state. It is obvious from Figures 3 and 4 of the present paper that the white pigment is in a concentrated condition at the same time as is the black.

It is possible on the basis of the observations recorded in Figures 1–4 to differentiate between the frequency of the diurnal cycle, which appears to be independent of the temperature of the environment, and its quantitative expression (the amplitude), which is definitely influenced by temperature. Although the rhythm was abolished at the end of 6 days for the black chromatophores in the animals maintained at 6° C., an examination of the figures shows that, as long as the amplitude is sufficient to indicate significant differences in chromatophore index, the frequency of the rhythm remains unchanged. Furthermore, since the white pigment still exhibited its diurnal rhythm at the end of 2 weeks, the underlying mechanism determining the frequency of the rhythm was obviously still operating.

In view of the data in these figures, one of two possible conclusions seems indicated: either external forces of a cyclical nature, other than light, temperature, or humidity, directly control the fre-

quency in *Uca*, or the determination of the frequency is endogenous and exhibits a temperature coefficient of 1.0.

The possibility of cyclical environmental forces as the agents effecting the rhythm in certain animals has been discussed in some detail by Park (1940). The fact that diurnal rhythms which have been artificially reversed have been shown to persist for some time under constant conditions subsequent to the reversal might at first suggest that control does not lie in an external factor. Such a factor would presumably recur once every 24 hours in its role as stimulator. In reversed rhythms the effective stimulus would, therefore, be 12 hours before or after the phase of the cycle normally induced by it. If, however, the external factor acts only as a generalized, nonspecific stimulus, with the direction of the response dependent upon the point in a series of reactions at which the stimulus occurs, then the possibility of external control must be admitted. If we are to explain the maintained diurnal rhythms of these animals in terms of external stimuli, however, a diurnally rhythmic factor other than light, temperature, or humidity must be responsible.

The results of the experiments in which the 24-hour rhythm is found to be thrown 6 hours out of phase with the normal day-night cycle after subjection of the animals to 0°–3° C. temperature for 6 hours clearly demonstrate a dependence of the normal rhythm in *Uca* upon metabolic processes. It also clearly proves that specific events in the rhythm are not determined directly by specific external events either directly or inversely, as discussed in the preceding paragraph.

If, however, it is postulated that the normal rhythm is maintained exclusively by a series of metabolic events proceeding at a relatively precise rate, one seeks in vain for descriptions of a biological mechanism which could reasonably be expected to maintain the rhythms in all the conditions under which they have been observed. Such a mechanism requires a process unaffected by temperature, a temperature-compensating mechanism, or a temperature-regulating mechanism. Such mechanisms for *Uca* are entirely hypothetical; but, even with the existence of such a one, it is difficult to credit it with sufficient precision to account for the remarkable synchronization of the rhythm of *Uca* for as long as a month in constant darkness at such a wide temperature range.

The possibility that some diurnally rhythmic factor in the external environment is operating cannot yet be entirely ruled out. Such a rhythmical event may possibly serve as a pacemaker for a series of metabolic events within the organism, which constitute the biological basis for the rhythm. Against such a concept is the difficulty of postulating how an external diurnally varying event can maintain a 24-hour rhythm in an organism despite the fact that the latter is no longer in phase with it. Furthermore, the *Uca* which were out of phase by 6 hours in the experiments reported in this paper showed no tendency to return to the normal day-night synchronization during the 6 days that they were observed. In support of a hypothesis of an external factor influencing the cycle is the apparent specificity of the 24-hour interval within the normal organism. For example, in time-training of bees, the specific time at which they are trained to approach the feeding station is obviously learned by the individual animals, and therefore the phenomenon might be classed as "habitual" rhyth-

micity. When one considers, however, that the animals cannot be trained on other than a 24-hour cycle, it is clear that this particular expression of rhythmicity must operate within the framework of a cyclic process previously established in the individual. It seems possible that the diurnal color changes observed in *Uca* may likewise represent only one expression of a basic metabolic rhythmicity.

A comparison of the differences between day and night phases of the black chromatophores under different conditions of temperature, as shown in Figure 5, emphasizes the influence of low temperatures on the degree of concentration or dispersion of the pigment. It is known that a hormone from the sinus gland is responsible for the dispersion of black pigment in *Uca*, and it is also possible that a concentrating substance is responsible for the night phase of the chromatophores. Even if one hormone is involved, a decrease in rate of either its secretion or its destruction, such as would be expected at low temperatures, would reasonably explain the observed condition. It is to be noted that the process involved here is one which agrees well with other commonly observed biological processes, in that the temperature coefficient is considerably greater than 1.

SUMMARY

1. The diurnally rhythmic color change observed in *Uca* has been shown to persist for as long as 30 days under conditions of constant darkness, constant temperature, and constant humidity.

2. It has been established that the white chromatophores of *Uca* exhibit a diurnal rhythm to the same extent and in the same direction as do the black chromatophores.

3. The frequency of the rhythm of both black and white chromatophores has been shown to be unaffected by temperature in the range from 6° to 26° C.

4. Evidence is presented which indicates that when all metabolic processes are greatly reduced in rate by temperatures approaching 0° C., the diurnal rhythm is delayed by an interval approximately that of the time of exposure to the low temperature.

5. A rhythm which has been delayed for approximately 6 hours appears to continue in constant darkness as a 24-hour rhythm, 6 hours out of phase. This has been observed to continue unmodified for at least 6 days.

6. It is concluded from the foregoing that the frequency of the rhythm must, at least in part, be metabolically determined.

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INGESTION OF COLLOIDAL MATERIAL AND WATER BY GOLDFISH[1]

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THE senior author has long been concerned with a series of studies on group physiology, with particular reference to aspects of the general sociology of nonhuman animals. In addition to joint research reports, summaries of progress and of closely related literature have been published from time to time (e.g., Allee, 1931, 1938, 1947). Considerable attention has been given to changes that animals produce upon the environment in which they live and to the effects of such a conditioned environment upon later inhabitants.

Within limits, the common goldfish (*Carassius auratus*) grow faster in reproducible artificial pond water, conditioned by placing other animals therein, than in unconditioned water. In part the increased rate of growth results from the presence of minute bits of regurgitated food in the conditioned medium (Allee, Oesting, and Hoskins, 1936; Allee, Finkel, and Hoskins, 1940; Allee, Frank, and Berman, 1946). The same effect is produced by the direct addition of similarly tiny food particles without allowing them to pass through the alimentary canal of conditioning fishes (Livengood, 1937).

Assay goldfishes isolated in either the fish- or the food-conditioned water grow better than accompanying controls, even though the supplementary nourishment provided by such water consists only of food particles of about 44 μ or less in diameter. The larger masses, including feces, are removed by passing the conditioned water through a phosphobronze sieve with 325 meshes to the inch. It was found some years ago that fish could ingest charcoal particles of this size range (Allee *et al.*, 1936), but the mechanism of ingestion was unknown. The growth-promoting effect of the medium is present to a reduced extent when the conditioning is done by starved fish.

Extracts from goldfish or from bullhead skin (*Ameiurus melas*) are also growth promoting for isolated goldfish.

[1] This investigation was supported in part by a grant from the Rockefeller Foundation. We are indebted to Dr. Asher J. Finkel and to Dr. Harold R. Koff for permission to report unpublished results obtained under the supervision of the senior author. We also thank Dr. O. H. Robertson, Department of Medicine, University of Chicago, a student of the biology of fishes, for suggesting an X-ray attack using thorium dioxide and for making available certain X-ray facilities; Dr. Paul C. Hodges for helping set up the X-ray technique used and for checking our reading of the pictures obtained; and Dr. Franklin C. McLean for placing his X-ray apparatus at our disposal.

These extracts are largely protein in nature and are effective when diluted to 1 part in 400,000, or even to 1 part in 800,000. The extracts were dissolved in an alkali solution and tested in comparison with suitable controls. Again the mechanism of intake and the whole mode of action remained unknown.

It was recognized from the beginning that these experiments in population physiology involved a phase of the more general problem of the utilization of dissolved materials or of colloidal particles. Many such relations are commonly considered in connection with Pütter's theory of parenteral animal nutrition, which first became prominent in 1909 and was restated by him in a series of papers, notably one in 1922, in which he gave a brief summary of the evidence to that date. Consideration of the subject dates back at least to the pioneering suggestion by Knörrich (1901). Krogh (1931) and Bond (1933), both of whom worked directly with certain aspects of the problem, gave critical summaries of the available evidence. The existence of their papers makes unnecessary a detailed historical survey. The earliest studies did not differentiate clearly between dissolved substances and those in colloidal suspension. Pütter emphasized the nutritive value of those in solution and regarded the gills as a major avenue of entrance. Solutions of organic compounds with a concentration considerably higher than those found in nature can be utilized by tadpoles (Kříženecký, 1925, 1928*a*, *b;* Bonnet, 1927) and are ingested by way of the digestive tract.

This approach leads directly to the problem of the ingestion of water by fishes, a subject of much interest to those dealing with osmotic regulation as well as to students of nutrition. There is no doubt but that marine teleosts drink sea water in connection with maintaining osmotic balance. The intake is related to the fact that they live in a hypertonic medium (Smith, 1930). Schiemenz (1905), discussing how fish eat, reported that fresh-water fishes do not swallow water but only pass it through their gill slits and swallow almost dry food. The development of the subject can be suggested by some near or actual quotations.

Smith (1930, p. 498) says that the eel, *Anguilla*, when in fresh water does not swallow significant quantities as it does when in sea water:

> Numerous observations on *Cyprinus* and *Carassius* kept in fresh water tinctured with phenol red show that in most instances no water is swallowed within 24 to 48 hours. Ultimately the dye does appear in the gastro-intestinal tract, and becomes highly concentrated in the intestine by the absorption of water. But the fact that water is not swallowed in detectable quantities within the first 24 hours stands in marked contrast to the situation in marine fish which invariably "drink" within 2 to 3 hours after being placed in tinctured sea water. In spite of the failure of fish in fresh water to ingest water, the urine flow is much larger than in marine fish. . . . Since this water is not absorbed from the gastro-intestinal tract it must enter the body of the fish either through the oral or esophageal membranes or through the gills, probably through the first.

The same investigator (1932, p. 11) says that "the fresh-water teleost apparently does not drink water but absorbs it through the oral membranes or perhaps through the gills." And Krogh (1939, p. 133) cites Smith (1930) in support of the statement that "fresh-water fishes do not drink water."

Ellis (1937, p. 402), in a monograph dealing mainly with his investigation of stream pollution and its effects on fresh-water fishes, from his own experience supports Bond's conclusion that bony fishes have little or no permeability to a variety of substances. Ellis states very

briefly that, by using colloidal dyes, he found that

> many fresh-water fishes refuse to swallow water containing various types of effluents for some time after being placed in such water. . . . However, after varying intervals, usually 48 hours or more, fresh-water fish swallow some of the surrounding water even if not fed, suggesting that the water needs of the body are not entirely supplied through absorption by the external surface or the lining of the mouth. The swallowing of polluted water allows any of the injurious or toxic substances which the water may contain direct access to the mucosa of the gastrointestinal tract.

These toxic substances may be absorbed and so affect internal vital organs. Ellis recognizes a group of such substances that act after they have been absorbed "from water swallowed by the fish." Arsenic can be accumulated in this way, even from low concentrations, if the fishes are given sufficiently long exposure.[2]

Schuster-Woldan (1936) used X-rays in his approach to the problem of whether fishes swallow water. He quotes with respect the statement of Schiemenz (1905) "dass der Fisch nicht 'trinkt.'" Schuster-Woldan exposed two tench (*Tinca vulgaris*) and two carp (*Cyprinus carpio*) for 30 hours in a darkroom with one of each species in barium sulphate and the other in a similar suspension of this X-ray opaque material in association with dissolved sugar. The published plate shows the *Tinca* that had been exposed to barium sulphate alone, and one each of the carp and of the tench immediately following exposure to suspensions of this salt with sugar added. The carp has its gut well filled with engulfed barium sulphate, and the similarly treated tench shows some opacity in the gut. The tench in barium sulphate alone has a darkened surface from the settling of the barium salt but contains none in its alimentary tract. The barium sulphate also settled out of suspension and formed a thin layer on the bottom of the aquarium. The experiment indicates that, under the conditions, the two fishes tested in barium sulphate plus sugar ingested some of it within 30 hours' exposure.

This presents the background as we now know it for the problem concerning how minute food particles, about 44 μ in diameter or less, are ingested by goldfish. The present paper will deal with this subject only. Discussion of the possible nutritive value of various minute objects will be reserved for a subsequent report.

ANIMALS AND THEIR TREATMENT

Although a small number of preliminary observations has been made with other species of fishes, the data reported here deal with common goldfish of the type used in previous studies of the effect of conditioned water on the growth of these fish. The size range of the goldfish was from 3 to 5 cm. body length with tail omitted. All the goldfish came originally from the Grassyforks Fisheries, Inc., Martinsville, Indiana. They had been kept in the laboratory in stock aquaria of 50 or 100 gallons for several months. The fish in these aquaria were fed daily, usually in recent years on a mixture of cooked corn and soy-bean meal, supplemented at irregular intervals by raw ground liver and by enchytraeid worms.

In early charcoal and dye tests, synthetic pond water constituted the experimental medium; all later experiments were made in tap water that had been

[2] Ellis, replying to an inquiry in 1948, confirmed his published report, saying, in addition, that he and Dr. Dallas K. Meyer of his department have accumulated further evidence that fresh-water fishes take water into the alimentary tract during the first 24–48 hours after transfer from one tank to another, apparently as a result of disturbance or excitement.

passed through a filter of activated charcoal to remove chlorine and other possible contaminants.

For the tests with dyes, stock solutions of various acidic, basic, and substantive dyes (Bancroft, 1932) were prepared in water or alcohol in a concentration of 0.1 per cent. These stock solutions were further diluted with the experimental medium to the concentrations given in Table 1. The charcoal used in ingestion tests was carefully ground in a mortar, so that much would pass through Whatman No. 3 filter paper; such a filtered suspension of carbon particles was used directly. In addition, a few essays were made in which India ink was added to the medium, making it possible to employ carbon particles of still smaller size. Larger particles with relatively little

TABLE 1

DYE INTAKE BY GOLDFISH*

Dye	Percentage Concentration	Hours of Exposure†		
		Up to 24	Up to 48	More than 48
Janus green	0.0005	Dead	.....	.....
	0.0001	Dead	.....	.....
Orange G	0.0005	----	--	.....
Dahlia	0.0002	-	-	-
	0.0001	-	-	-
Acid fuchsin	0.002	-	-	-
	0.001	--	--	--
	0.0005	-	-	-
Methyl green	0.0007	-	-	-
	0.00035	-	-	+
Aniline blue	0.002	--	--	-
	0.001	---	---	-++
	0.0005	--	-	+
Nile blue	0.001	Dead	.....	.....
	0.0005	-	-	-
	0.0002	--	--	+
	0.0001	--	-+	+
Trypan blue	0.001	--	-+	+
	0.0005	----	--+	+
Congo red	0.0005	--------	-----+	-++
	.....	--++++++	+++++	.....
	0.0001	----+	-++++	++
Cresyl violet	0.0005	--	++	+
	0.0003	-++	++	++
	0.00015	-+	-	++
Bismarck brown	0.0005	+	.....	.....
Eosin	0.0005	+	.....	.....
Phenol red	0.0005	---+	.....	.....
Methylene blue	0.0005	---+	+++	.....
Naphthol green	0.0005	--++	--++	++
Carmine	0.002	++	.....	+
	.001	++	++	+

* See p. 385.

† Each − or + summarizes results on one fish during the designated time interval.

variation in size and with a maximum diameter of about 12 μ were also employed in an attempt to analyze the mechanism of ingestion. Such material was easily obtained from cultures of the alga, *Chlorella vulgaris*, passed through a Sharples supercentrifuge at 35,000 r.p.m., and resuspended in filtered water.

The results of the tests with dyes, charcoal, etc., were evaluated by examining the feces of the fish under a dissecting microscope. The fish were removed from the medium containing the dye and were placed in fresh clean water. In nearly all cases feces eliminated into this medium rather than into the dye suspensions were inspected. Some examinations of fish were also made following dissection to allow inspection of the digestive tract.

In addition to these tests, a number of fish were exposed for varying lengths of time to suspensions of colloidal thorium dioxide in an aqueous solution of dextrin. The suspension, called "Thorotrast," is manufactured by Heyden Chemical Corporation, New York. The range of particle size, determined by using the electronic microscope, is 3–10 millimicra with 7 millimicra as the average size. The particles are irregular in shape and probably flat.[3] Thorotrast was used in concentrations ranging from 0.67 to 4.0 per cent. In these, as well as in a number of the experiments with dyes, galvanized metal screens with quarter-inch meshes were placed some distance above the floor of the experimental containers, so that the fish could not ingest feces or other material collected on the bottom.

Most fish were starved for various periods before being exposed to the Thorotrast or were handled immediately before exposure. One group of later tests employed fish that had not been handled and had had food (enchytraeids) continually available to them for 8 days previous to the X-ray. In the cases in which the fish had not been handled until the X-ray, the Thorotrast was simply poured into the water around them. At the end of the designated period of exposure, the fish were anesthetized with chloretone and were X-rayed. Different individuals were used for each test.

[3] Personal communications from Mr. J. P. Remensnyder, Heyden Chemical Corporation.

EXPERIMENTAL RESULTS

The results obtained from the series of experiments with dyes are summarized in Table 1. Some of these studies have been repeated by others, with results substantiating the essential observations. In many instances no feces were eliminated during the test periods, and these fish are not included in the table. Only in those cases in which the feces of a particular fish were found to contain dye material inside the gelatinous covering was the result designated with a plus sign, all other evidence being regarded as negative. Each symbol within one time class represents the results obtained from one fish, but observations made on the same individual fish may be recorded in different columns.

The evidence shows that, whereas dye is eventually present in the feces of the majority of the fish tested, a much smaller percentage show dye in the feces after 24 hours. Since, however, no evidence was obtained on the time required for dye to pass through the entire digestive tract in the fish, which were not taking in food in that period of time, no definite conclusions can be drawn from these tests as to the time after initial exposure when the fish ingest the dye. There are strong suggestions, as in the data obtained with Congo red, that individual variability exists.

Parallel experiments with charcoal, India ink, and algal suspensions, of the

TABLE 2

EVALUATION OF X-RAY DATA FROM FISH EXPOSED TO SUSPENSIONS OF COLLOIDAL THORIUM DIOXIDE

I. STARVED FOR 24–72 HOURS PREVIOUS TO ADDITION OF THOROTRAST

Specimen	Hours in Thorotrast	Percentage Concentration	Result*
	a) Handled Immediately Previous to Addition		
1	96	1.3	+
2	96	1.3	++
3	72	2.6	++
4	72	1.3	+
5	24	1.3	++
6	24	1.3	++
7	24	1.3	+++
8	24	1.3	++++
9	24	0.67	+
10	24	0.67	±
11	6	1.3	+
	b) Not Handled for 24–73 Hours Previous to Addition		
12	24	4.0	++
13	24	1.3	+++†
14	24	1.3	++++
15	24	1.3	+++
16	24	1.3	++
17	24	1.3	++
18	6	1.3	+++
19	5	1.3	++++‡
20	1	1.3	++
21	1	1.3	−

II. HANDLED PREVIOUS TO ADDITION NOT STARVED

Specimen	Hours in Thorotrast	Percentage Concentration	Result*
22	72	1.3	++
23	72	1.3	++
24	24	1.3	++++
25	1	1.3	+
26	1	1.3	+
27	1	1.3	±
28	1	1.3	−

III. NOT HANDLED OR STARVED FOR 8 DAYS PREVIOUS TO ADDITION

Specimen	Hours in Thorotrast	Percentage Concentration	Result*
29	24	1.3	+
30	24	1.3	++
31	24	1.3	+++
32	24	1.3	+++
33	5	1.3	+++

* Each line summarizes results from one fish. The number of + signs here indicates the amount of Thorotrast in the gut.
† Fig. 3.
‡ Fig. 1.

same order of density as the dyes, gave positive evidence that such particles are ingested; the presence of carbon or algal cells in the feces was unmistakable. Dissected animals showed the dyes, carbon, or algal particles in the gut. The oral cavity was nearly always free from particles or dye adhering to the mucus. In one or two cases traces of dye could be detected in the mucus, but these were not sufficient to explain the quantity in the gut itself unless an extraordinary amount of concentration occurred there. The only evidence favoring a concentrating mechanism in the mouth region was obtained from the larger alga cells, which were collected on the gill rakers in significant amounts.

Tests with colloidal thorium dioxide give evidence that particles are ingested, and they suggest, in addition, that the particles are ingested with the water in which they are suspended. Table 2 summarizes the results obtained. The evidence from each fish was graded in accordance with the amount of contrast obtained. A plus-minus sign (±) indicates cases in which the presence of Thorotrast is doubtful. Four plus signs represent the maximum contrast obtained, illustrated by Plate I, Figure 1. Since the X-ray techniques were fairly

well standardized, except in the first tests, the degree of contrast should represent an approximation of the amount of thorium dioxide present in the gut. Detectable ingestion of the material sometimes occurs in as little as 1 hour; and in all tests of longer duration, with the possible exception of a trial using a lower concentration of the contrast medium, the results were clearly positive. In none of the fish could any traces of the thorium dioxide be detected in the mouth cavity by the X-ray method. In the fish in which positive results were obtained from 1 hour's exposure and in one fish exposed for 6 hours, the contrast medium outlined the walls of the gut and was perhaps adsorbed onto the wall. In other instances, the Thorotrast formed a solid line, ranging from the bare limits of visibility to the thickness shown in Plate I, Figure 1. All nine control fish tested were distinctly negative, closely resembling Plate I, Figure 2.

The two treated fish showing no opacity in the gut had each been placed in 1.3 per cent concentration of Thorotrast for 1 hour, one with and one without having been handled immediately before the exposure. Of the two doubtful cases (±) both fish had also been handled as they were being introduced into the Thorotrast. One had been in a 1.3 per cent suspension for an hour's time, and the other had spent 24 hours in a concentration of 0.67 per cent. With regard to each doubtful or negative instance, one or more other fish yielded definitely positive results under similar treatment.

DISCUSSION

Full agreement is lacking in the literature regarding whether fresh-water fish drink water. Our immediate impulse to investigate this subject arose from the need to understand how goldfish derive nutrition from water containing food particles of about 40 μ in diameter or smaller. Initially, we attempted to find some mechanism in connection with collection on mucus or by some other oral device. We knew that gill rakers are said to aid in the concentration of plankton, as Hesse and Doflein (1914) say they do in the tench (*Tinca vulgaris*).

Our search for collecting mechanisms steadily yielded negative results so far as small particles were concerned. In one or two instances faint traces of methylene blue were found in the mouth. Even considering the ability of fish to concentrate materials in the intestine, the small traces of this one dye found on oral mucus does not furnish proper support on which to base an explanation of the collections that goldfish are known to make. No traces of carbon particles, colloidal dyes, or Thorotrast were found in the mouths of goldfish.

All the evidence known to us can be explained by the logical assumption, based on the work with dyes and with Thorotrast, that goldfish swallow considerable quantities of water. Apparently, it is safe to make the generalization that many other fresh-water fishes also have this habit (Ellis, 1937). The limited X-ray tests made by Schuster-Woldan (1936), if accepted as reported, point toward the same conclusion when sugar was also present. Perhaps the barium sulphate settled out of suspension more slowly in the presence of sugar and so allowed better opportunity for its ingestion.

Schuster-Woldan reported tests on four individuals only, two carp and two tench. He asserted, but by no means proved, that the two fishes in the sugar solution swallowed water and the contained barium sulphate because of taste stimulation by the sugar. Our experi-

ments with colloidal thorium dioxide do not give critical evidence on this point, since Thorotrast contains dextrin. Such a suspension conceivably may have stimulated goldfish to swallow water, but the rather extended series of frequently positive results with dyes and carbon particles, all placed directly in the water, do demonstrate that goldfish swallow unsweetened water.

Available data give no suggestion concerning how much water goldfish swallow or how frequently they do it. Certain of the observations of Ellis (1937) imply the swallowing of a considerable amount of water by some of the fishes with which he worked.

The source of stimulation causing fresh-water fishes to drink water remains unknown. Smith (1930, p. 499), in discussing marine fishes, reports that handling or removing them in a net leads to increased ingestion of water. The experiments with Thorotrast were devised, in part, to test this point. Five individuals were not handled for a period of 8 days immediately before the addition of Thorotrast; all contained quite distinct amounts of thorium dioxide, one after being in the Thorotrast for 5 hours, and the others after 24 hours' exposure.

Ten other goldfish were not handled for periods ranging from 24 up to 73 hours before the addition of Thorotrast. X-ray exposures showed that nine of them had engulfed this substance, one after swimming in it for only 1 hour, one for 5 hours, and another for 24 hours. In one instance no trace was found after an hour in the Thorotrast. For comparison, inspection of Section II of Table 2 will show that, of four other fish handled just before being put into the Thorotrast, an hour later one had not engulfed this material, another showed a trace of opacity in the gut, and the remaining two contained definite amounts.

Both X-ray photographs of fish containing Thorotrast in the gut that were selected for exhibit in Plate I were of individuals that had not been handled immediately before being exposed to X-ray opaque material. In general, the individuals that were not handled (Table 2) made as good a showing as those that were.

The experiments with intake of dyes, summarized in Table 1, were not so closely timed. Fewer of the fish tested showed dyes in their feces before 24 hours' exposure than at later periods. All the individuals tried had engulfed substantive dyes before an exposure of 72 hours.

Certain of the experiments, both with dyes and with Thorotrast, were carried on with the fish in glass fingerbowls (10 cm. in diameter); in others the fish were kept in opaque earthenware jars (12.5 cm. in diameter). Often two individuals were present together. We do not know the extent to which the fishes, bumping into each other or against the walls of their containers, particularly of the fingerbowls, would produce increased stimulation. There was no particular evidence that the animals were unduly stimulated and no evidence that swallowing water was affected by stimulation within the range that we tested.

As has been stated before, these experiments were initiated in an attempt to understand the method of intake of minute nutritive particles from homotypically conditioned water. In the course of routine experiments during the past several years, the assay fish were daily isolated in 1 or 2 liters of water in earthenware jars, allowed to remain undisturbed for approximately 21 hours, fed by introducing appropriate food, and then transferred to fresh medium in another jar. We have no indication concerning when the assay fish began swallowing water, except that no distinction

in growth was apparent for fish fed in conditioned water as contrasted with those transferred to uncontaminated water and immediately fed there (Livengood, 1937). We have strong doubts whether, under the conditions of the whole series of experiments with conditioned water, goldfish swallowed water primarily because of recent stimulation.

Apparently, the contrast between the ready drinking of water by marine fishes and the reported nondrinking by freshwater fishes is not so sharp as some statements by Smith (1930, 1932) and by Krogh (1939) had led us to believe. It is true that the latter (1931, p. 438) states that "it seems legitimate therefore to conclude that the absorption of nutrient substances observed in Kříženecký's and Krogh's experiments must have taken place through the intestine. It is commonly asserted that aquatic animals take in their food without water, but it is evident that this assertion is untenable." Our own work supports this summary, and all the available evidence tends to indicate that there is not a universal break in drinking habits between freshwater and marine fishes. We have no direct information concerning the quantitative difference in this respect between fishes from these two major aquatic habits.

SUMMARY

1. In keeping with indications from the work of others, goldfish engulf dyes in solution and in suspension. Minute carbon particles, including finely ground charcoal and India ink, are also accumulated in the intestine.

2. Colloidal thorium dioxide (Thorotrast) with a mean particle size of 7 mμ was taken into the intestine, frequently in large quantities, as shown by X-rays.

3. No trace of the Thorotrast was found in the mouth. Only one of the several dyes tested (methylene blue) produced observable coloration in the oral region, and that one gave only a faint trace of color.

4. Thorotrast could be detected in the intestine after the fish had spent as little as 1 hour or as much as 96 hours in the colloidal suspension. These were the shortest and the longest exposures tested.

5. Thorotrast was engulfed with or without previous starvation and with or without the goldfish being handled before exposure to this X-ray opaque material.

6. The observations give strong evidence that goldfish swallow water and the substances it contains; in short, that they drink fresh water.

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PLATE I

Photographs of X-ray films of goldfish following their exposure to colloidal thorium di xide.

FIG. 1.—After 5 hours in 1.3 per cent Thorotrast; see No. 19, Table 2.

FIG. 2.—Control.

FIG. 3.—After 24 hours in 1.3 per cent Thorotrast; see No. 13, Table 2.

STUDIES ON THE RESPIRATION OF AN AMOEBA, MAYORELLA PALESTINENSIS

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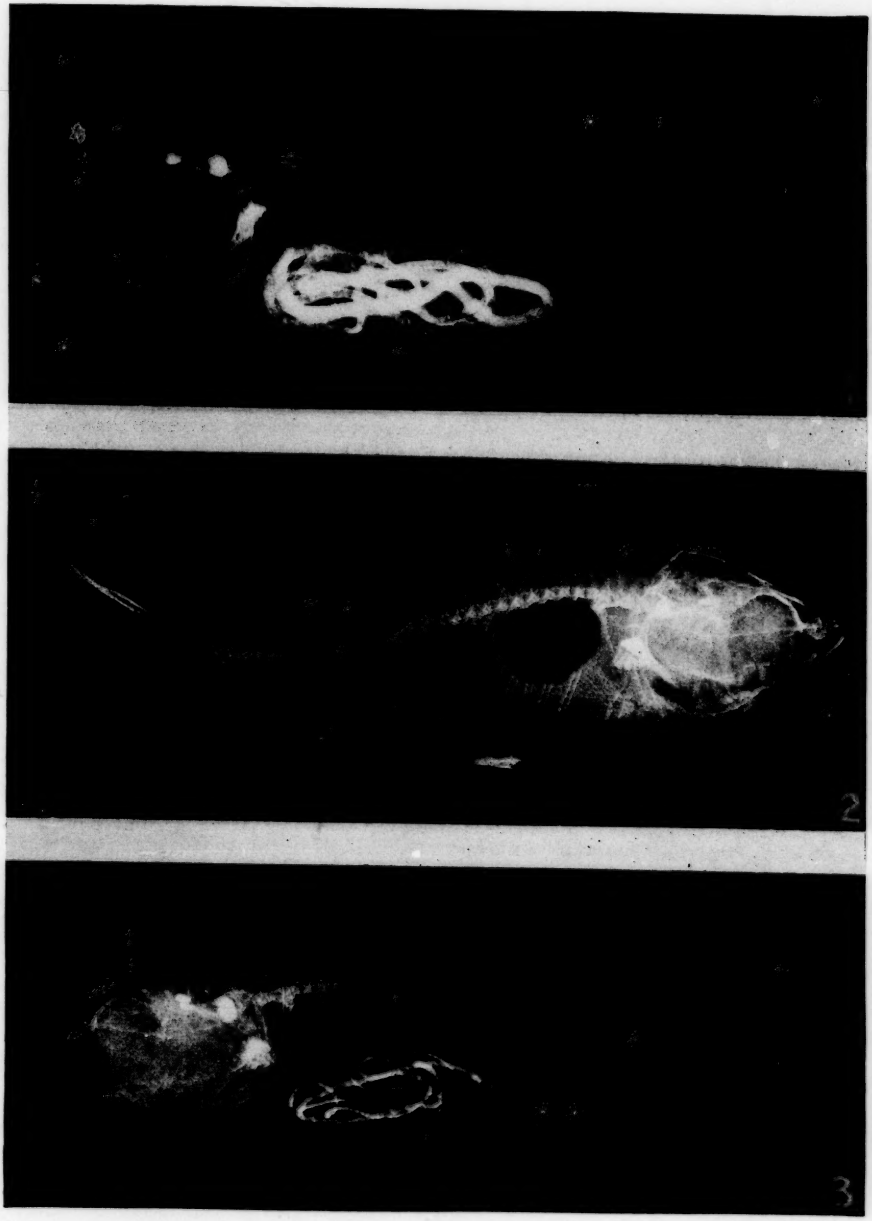
A PERUSAL of the review given by Jahn (1941) on respiration of Protozoa reveals that, although cells of Protozoa represent an excellent subject for physiological research, only little work has been done on their respiration. The main reason for this may be found in the fact that, as yet, it has been possible in only a few cases of heterotrophic Protozoa to obtain bacteria-free cultures. On the respiration of Rhizopoda there exist only three papers: two older ones, by Emerson (1929), who worked with *Amoeba proteus*, and by Howland and Bernstein (1931), who measured the respiration of isolated *Actinosphaerium eichhornii* cells. In both cases the investigations were carried out on cultures containing bacteria and in distilled water, both factors which make the reliability of the results rather questionable. More recently a paper was published by Pace and Belda (1944) on the respiration of *Pelomyxa carolinensis*. In this case, too, the cultures contained bacteria, and the amoebae were thoroughly washed in

PLATE I



sterile buffer solution before the experiment.

For these reasons it seemed desirable to investigate the respiration of amoebae with more modern methods, especially as bacteria-free cultures of *Mayorella palestinensis* Reich have been grown in our department for some time.

MATERIAL AND METHODS

The amoebae used in these experiments were grown in test tubes, each containing 4 ml. of the previously described culture solution (Reich, 1935), consisting of NaCl, 0.1 per cent; K_2HPO_4, 0.01 per cent; $MgSO_4$, 0.01 per cent; $CaCl_2$, 0.01 per cent; peptone, 1 per cent; glucose, 1 per cent, at pH 6.2. For each experiment seventy to one hundred cultures, approximately 3 weeks old, were centrifuged, washed three times with a sterile solution of 0.15 per cent NaCl, and suspended in a fresh nutrient solution. The concentration of the amoebae was determined by direct count in the haemacytometer. In most experiments the above-mentioned culture solution was used either complete or omitting one or both of the organic components (peptone, glucose). In other series of experiments this nutrient solution was replaced by a salt solution composed of 0.15 per cent NaCl, 0.005 M KH_2PO_4–NaOH buffer with or without peptone and glucose.

All measurements were made with the help of a Warburg manometer at 27° C. As this technique has been repeatedly described in full (Dixon, 1943; Umbreit, Burris, and Stauffer, 1946), it is not necessary to give a detailed account here.

In order to avoid any possible error caused by ammonia which might be produced by the respiration of the amoebae, 0.2 ml. of a 0.3 M H_2SO_4 solution was added in the side bulb of the respiratory vessel, according to the method of Specht (1935).

For the determination of the respiratory quotient the method of three manometers was used, which made it possible to measure both the free and the retained carbon dioxide. As has been shown by the results, which will be discussed later, the usual methods of determination of the carbon dioxide production were unreliable, for in some of the solutions used in these experiments more than 50 per cent of the carbon dioxide formed was retained.

RESPIRATION IN THE NORMAL SOLUTION

The aim of the first series of experiments was to determine the respiration of *M. palestinensis* in the normal nutrient solution. A great number of measurements, 52 series of experiments with two to four manometers each—altogether, 116 single determinations—showed that 10,000,000 amoebae required, on the average, 110.8 mm^3 O_2 per hour at 27° C. When leaving out of consideration 10 series of experiments (including 20 single experiments), in which amoebae starved for 24 hours were used, we obtained an average of 117.3 ± 20.8 mm^3 O_2/hour/10^7 amoebae, the different measurements varying between 83 mm^3 O_2/hour/10^7 amoebae and 170.0 mm^3 O_2 hour/10^7 amoebae, and a mean respiratory quotient of 0.867. Details of these measurements will be discussed later.

Pace and Belda (1944) found that *Pelomyxa carolinensis* requires 90.1 mm^3 O_2/hour/10^7 amoebae at 25° C. and 132.4 mm^3 O_2/hour/10^7 amoebae at 30° C. The far-reaching agreement of their results with those obtained for *M. palestinensis* is all the more striking as the volume of the *Pelomyxa* cell exceeds many times that of the *Mayorella* cell.

In spite of all precautions, it was im-

possible to exclude fluctuations in the results. Smaller variations occurred even when identical material was used. Thus Table 1 shows the results of four experiments which were carried out on four consecutive days with cultures of the same series and in simultaneously prepared nutrient; in each of these experiments two manometers were used. The relatively small fluctuations shown in Table 1 are certainly due to inaccuracy

TABLE 1

RESULTS OF EXPERIMENTS ON THE RESPIRATION OF AMOEBAE IN COMPLETE NUTRIENT SOLUTION

Carried out on four consecutive days with the identical nutrient solution and identical series of cultures.

Experiment No.	No. of Amoebae	Rate of Respiration O_2 mm^3/Hour/10^7 Amoebae		
		Manometer 1	Manometer 2	Mean of Two Manometers
1	10^7	111.3	119.3	115.3
2	10^7	130.8	129.3	130.0
3	1.2×10^7	110.2	118.6	114.4
4	$. 10^7$	133.3	133.3	133.3
Mean of four experiments				123.2 $\sigma = \pm 9.8$

in counting the amoebae and to variations in the temperature which amounted to $\pm 0.^{\circ}2$ C., whereas the much greater differences which appear in the results of experiments with different cultures may have been caused by the different physiological state of the amoebae. It thus appeared impossible to obtain complete conformity in the various experiments dealing with the influence of external conditions on the respiration of amoebae. Therefore, parallel control tests were made in each experiment; the results of the experiments were expressed as percentages of the respiration of the controls and not as absolute values.

Attempts have often been made to relate the amount of respiration of the protozoan cell to the growth conditions and age of the culture and to the concentration of the cells in the manometer. Pace and Lyman (1947) in *Tetrahymena geleii* found a direct relation between the concentration of the cells in the manometer and the oxygen consumption. No such correlation could be found for *M. palestinensis*. It appears most unlikely that such a correlation does exist, especially when the results obtained with other types of cells and tissues are taken into consideration. It might rather be assumed that in the experiments of Pace and Lyman the manometers were not shaken sufficiently quickly to produce an equilibrium between the gas and the liquid phase.

Wachendorf (1912) has already shown for *Colpidium colpoda* (=*T. geleii?*) how much the age of the culture influences the respiration: it amounted to 191.0 mm^3 O_2/hour/10^6 organisms for 1-day-old cultures and decreased to 151.0 $mm.^3$ O_2 for 10-day-old cultures and to 59.0 $mm.^3$ for 50-day-old cultures. Similar results were obtained by Peters (1929) for the same species: 200 mm^3 O_2/hour/10^6 organisms for growing cultures and 50 $mm.^3$ O_2 for old cultures. These results were confirmed in recent investigations by Baker and Baumberger (1941) for *T. geleii* and especially by Ormsbee (1942) in the same species. Ormsbee found, in experiments with a 2 per cent peptone solution in 0.05 M phosphate buffer, an oxygen consumption of 433.0 mm^3/hour/10^6 organisms in the exponential phase of the culture growth and 633.0 mm^3 O_2/hour/10^6 organisms in the stationary phase.

As a *T. geleii* cell is larger in the stationary phase than in the exponential,

the proportion is reversed when the respiration is expressed in terms of weight units and amounts to 81.1 mm^3 O_2/hour/mg in the exponential phase and 77.7 $mm.^3$ O_2 in the stationary phase. When this solution was replaced by a buffer solution free of peptone, the corresponding figures were 26.0 mm^3 O_2/hour/10^6 organisms in the exponential phase and 16.5 mm^3 O_2/hour/10^6 organisms in the stationary phase, or 146.2 mm^3/hour/mg in the exponential and 133.7 mm^3 O_2/hour/mg in the stationary phase of the growth of the culture. I shall return later to these striking differences between the results with different solutions when discussing my own results for the respiration of *M. palestinensis* in solutions free of nutritive substances. Pace and Lyman, too, in a recent investigation (1947) of the respiration of *T. geleii* found that ciliates from old cultures use much less oxygen than do those from younger cultures.

For Protozoa other than *Tetrahymena* the influence of the age of the culture on respiration was described by Riedmueller (1936) in *Trichomonas foetus* and by Hutchens (1941) in *Chilomonas paramecium*. But in all these cases the authors were dealing with rapidly growing cultures which reached the stationary phase after 24–48 hours, whereas *M. palestinensis* grows very slowly under the culture conditions described and reaches its stationary phase only after 3–4 weeks. As the formation of cysts begins before this phase is reached, only cultures which were still in the exponential phase of their development but were approaching their maximum density of population were used, i.e., cultures approximately 3 weeks old.

The figures given by Ormsbee show clearly that in many cases the interpretation of results depends on whether the results are expressed in terms of oxygen consumption per number of cells or per unit weight. Pace and Kimura (1944) have shown that in a comparison of the respiration of two species of *Paramecium* (*P. aurelia* and *P. caudatum*) the figures differ when referred to the number of cells but they are nearly equal for both species when expressed in terms of unit of volume. This question has often been discussed recently but without any definite results (cf. the review by Jahn, 1941). Tang (1941) points out that the structural and physiological differences between the various types of cells are so great that it is hardly possible to find a common unit of comparison for their respiration rate.

A method which has sometimes been employed consists in determining the volume of the centrifugal sediment; however, this involves so many sources of error that it cannot provide comparable results.

Only for certain Ciliata, viz., species of *Paramecium* with their relatively constant shape, does the determination of the volume of the cell by measuring both cell axes seem adequate. But it is impossible to use this method in dealing with flattened amoeba cells containing many vacuoles and sending out numerous pseudopodia. Pace and Belda (1944) succeeded in determining the volume of the relatively large *Pelomyxa* cells by sucking up single amoebae into glass capillaries of known diameter. They thus determined the total volume of the cells used in each experiment. They also tried to find a relation between the cell surface and oxygen consumption. But this method, too, is probably least suitable for amoeboid cells.

There remain only two possibilities: to determine either the number of cells or their weight. Both methods may lead to false conclusions; but the former has the advantage that the suspended cells are

easy to count. Therefore, wherever there is a question of comparing cells of a homogeneous population, this method seems preferable. The alternative of determining the weight of dried cells and of their nitrogen content has the disadvantage that it involves a great amount of material and time. Its reliability, on the other hand, is only apparent, as it is impossible to know in each case how much of the weight is constituted by the living protoplasm of the cell and how much by food and reserve substances or possibly by skeletal structures.

In order to facilitate the comparison of the respiration of *M. palestinensis* to the respiration of other Protozoa, a determination of the weight was carried out in one case. Ten million amoebae, washed in distilled water and dried at 100° C., weighed 11.6 mg. and had a nitrogen content of 10.7 per cent of their dry weight. Incidentally, this nitrogen content agrees well with the value of 10.6 per cent found by Ormsbee (1942) for *T. geleii*.

On the basis of this weight determination, the average respiration per 10 mg. of dried amoebae can be calculated: it amounts to 101.1 mm^3 O_2/hour/10 mg. A comparison with the figures found by earlier investigators for *T. geleii* (which were reduced by Ormsbee [1942] to terms of weight unit, cf. his Table 5) shows that the respiration rate obtained for *M. palestinensis* agrees well with most of the figures found for *Tetrahymena*. However, Pace and Lyman (1947) found much higher values for *T. geleii*. Similarly, the values already mentioned which Ormsbee found for the respiration in a peptone solution are much higher, while the value given by Lwoff (1934) is approximately intermediate between these and the values of other authors. Jahn's summary (1941) of the data for other protozoan species (cf. his Table 4) shows that even the results for one and the same species, such as *P. caudatum*, vary so widely that they do not permit a definite conclusion. The more recent investigations by Pace and Kimura (1944) on two species of *Paramecium* do not help to clear up this problem either. Hutchens (1941) found distinct differences in respiratory intensity between his two strains of *C. paramecium*.

It becomes more and more evident that in the present state of our knowledge it is not possible to find a common base of comparison for the results obtained with different species or even strains of the same species. This is not surprising when it is considered that Kidder and Dewey (1945) found considerable difference in the metabolism of various strains of *T. geleii* which they grew in pure culture. In order to get some idea of the metabolism of the cysts, their respiration was studied in one experiment. For this purpose, older cultures, in which most of the amoebae were encysted, were centrifuged. In order to kill the remaining amoebae which were not yet encysted, the centrifugal sediment was left in 1 per cent HCl overnight; the next day, the acid was neutralized with NaOH, and the cysts were washed three times with fresh salt solution. Finally, they were suspended in fresh nutrient solution, and 2 ml. of the suspension containing 90,000,000 cysts were transferred to a manometer. In this experiment the consumption of oxygen was 22 mm^3 O_2/hour/10^7 cysts, or approximately 20 per cent of the respiration rate of the amoebae. The R.Q. was 0.85—or about as much as in the metabolism of the amoebae. This would appear to indicate that the main reserve substances of the cysts are proteins.

INFLUENCE OF THE SUBSTRATE ON THE RESPIRATION

In the following section the results of experiments carried out in order to determine how the nature of the substrate influences the respiration are described. For this purpose the respiration in purely inorganic salt solutions was compared with the respiration in salt solutions to which either 1 per cent peptone or 1 per cent glucose or both had been added.

In order to distribute the amoebae equally to all manometers used, the cultures were centrifuged, washed three times with a pure salt solution, and suspended in a little over 4 ml. of the same salt solution. One ml. of this suspension was placed in each of the respiratory vessels; 1 ml. of the same salt solution was added to each of two manometers, and the respiration in these manometers was regarded as controls, while in two other manometers 1 ml. each of the solution under examination was added. In most of the experiments reported in this section a salt solution of the same composition as in the above nutrient solution was used. Only in the experiments summed up in Table 10 was it replaced by a 0.15 per cent NaCl solution with 0.005 M phosphate buffer of pH 6.0. In the experiments reported in Table 4, the usual salt solution with 1 per cent glucose served as a control, while in those given in Table 6 a solution containing 1 per cent peptone and 1 per cent glucose was used as a control.

INFLUENCE OF PEPTONE ON THE RESPIRATION

Tables 2 and 10 show the results of experiments in which the respiration in the salt mixture or in the NaCl buffer solution was compared with the respiration in a solution containing 1 per cent peptone and 1 per cent glucose. From these tables it can be seen that with the addition of peptone and glucose there is an increase in the respiration, varying from 8.1 to 19.6 per cent, and that, on the average, it amounts to +14.7 per cent ($\sigma = \pm 3.6$) in the salt mixture, while in the buffer solution it amounted, on the average, to +15.9 per cent ($\sigma = \pm 2.2$). There is, therefore, no real difference between the respiration in the two salt solutions used.

TABLE 2

OXYGEN CONSUMPTION IN SALT SOLUTIONS WITH AND WITHOUT 1 PER CENT PEPTONE AND 1 PER CENT GLUCOSE

Experiment No.	Oxygen Consumed ($mm.^3$)		Difference (Per Cent)
	Salt Solution Alone	With 1 Per Cent Peptone and 1 Per Cent Glucose	
1	154.0	174.2	+13.1
2	163.1	195.1	+19.6
3	181.0	207.0	+14.3
4	172.9	200.5	+15.9
5	150.9	174.9	+15.9
6	176.2	198.9	+12.8
7	168.1	181.8	+ 8.1
8	133.6	158.2	+18.4
Mean			+14.7 $\sigma = \pm 3.6$

Table 3 shows the results of a comparison between the respiration in the salt solution and that in a solution to which 1 per cent peptone had been added: the increase of the respiration, in this case, amounted to 8.3–16.9 per cent (on the average, +11.4 per cent, $\sigma = \pm 2.9$).

Finally (Table 4), the respiration in a solution of 1 per cent glucose was compared with that in a salt solution containing 1 per cent peptone and 1 per cent glucose, and the results of these experi-

ments showed an increase varying between 6.5 and 14.5 per cent (on the average +12.1 per cent, $\sigma = \pm 3.6$).

When the results of these four series of experiments are summed up, it can be seen that, when 1 per cent peptone and 1 per cent glucose are added, the respiration increased 8.1–19.6 per cent (on the average, 15.0 per cent), while, when peptone alone is added, this increase amounts to 6.5–16.9 per cent (on the average, 11.8 per cent). Considering the great fluctuations of the results obtained in various experiments, this difference seems hardly significant.

TABLE 3

OXYGEN CONSUMPTION IN SALT SOLUTIONS WITH AND WITHOUT 1 PER CENT PEPTONE

Experiment No.	Oxygen Consumed (Mm.³)		Difference (Per Cent)
	Salt Solution Alone	Salt Solution with 1 Per Cent Peptone	
1	210.0	227.6	+ 8.3
2	214.6	250.9	+16.9
3	166.1	183.3	+10.3
4	191.4	207.8	+ 8.5
5	188.7	208.4	+10.4
6	189.7	215.4	+13.5
7	193.1	213.5	+10.5
8	199.1	225.2	+13.1
Mean			+11.4 $\sigma = \pm 2.9$

TABLE 4

OXYGEN CONSUMPTION IN SALT SOLUTION CONTAINING 1 PER CENT PEPTONE AND 1 PER CENT GLUCOSE, AS COMPARED WITH SALT SOLUTION CONTAINING 1 PER CENT GLUCOSE

Experiment No.	Oxygen Consumed (Mm.³)		Difference (Per Cent)
	Salt Solution with 1 Per Cent Glucose	Salt Solution with 1 Per Cent Peptone and 1 Per Cent Glucose	
1	134.0	142.8	+ 6.5
2	232.1	254.1	+ 9.4
3	168.8	192.6	+14.1
4	187.6	215.7	+14.5
5	250.1	284.9	+13.9
6	224.2	256.6	+14.4
Mean			+12.1 $\sigma = \pm 3.6$

INFLUENCE OF GLUCOSE ON THE RESPIRATION

In a further series of experiments the influence of glucose on the respiration was investigated. Table 5 shows the results obtained when the respiration in the salt solution was compared with that in a solution to which 1 per cent glucose had been added. The difference varies between −1.3 and +1.7 per cent and was, on the average, 0.0 ($\sigma = \pm 1.16$).

TABLE 5

RESPIRATION IN SALT SOLUTIONS WITH AND WITHOUT 1 PER CENT GLUCOSE

Experiment No.	Oxygen Consumption (Mm.³)		Difference (Per Cent)
	Salt Solution Alone	Salt Solution with 1 Per Cent Glucose	
1	112.1	112.2	0.0
2	109.4	109.0	−0.3
3	168.5	168.4	0.0
4	168.9	170.5	+0.8
5	163.8	161.6	−1.3
6	160.7	158.5	−1.3
7	162.5	165.4	+1.7
Mean			0.0 $\sigma = \pm 1.16$

Table 6 contains the results obtained with a somewhat different method. In these experiments the respiration in a complete nutrient solution containing 1 per cent peptone and 1 per cent glucose was compared with a similar solution but without glucose. Although the results of

the various experiments in this series differed very much (they varied between −2.9 and +6.7 per cent), the average, +1.3 per cent ($\sigma = \pm 3.47$), is a figure which is still well within the limits of experimental error.

When finally calculating the average rate of oxygen consumption in all twenty-one experiments which were carried out within purely inorganic salt solution, using two manometers in each experiment, an average consumption of 101.6 mm^3 O_2/hour/10^7 amoebae ($\sigma = \pm 8.64$) was found. When this figure is compared with that of respiration in a normal nutrient solution of 117.3 mm^3 O_2/hour/10^7, it becomes evident that the rate of respiration increases by 15.4 per cent when 1 per cent peptone and 1 per cent dextrose are added. Thus it is possible to gain an additional confirmation of the results given in Tables 2 and 10.

The experiments on the influence of the substrate on the respiration may be summarized as follows (the relation between substrate and R.Q. will be discussed later):

a) There is no direct influence of glucose on the respiratory rate. This result is all the more striking as Reich (1936) found that in cultures of *M. palestinensis* the maximal density of population was three times higher when 0.5–1 per cent glucose was added than in glucose-free solutions. On the other hand, it was found earlier (Reich, 1935) that no acids are produced by the amoebae in cultures containing sugar. The present results are in favor of the assumption that glucose influences only the general environmental conditions but does not directly participate in the metabolism of the amoeba. Leichsenring (1925) found for *P. caudatum* that an addition of glucose caused an increase of the respiration of 7 per cent; and Emerson (1929) found under similar conditions an increase of 10–20 per cent for *Blepharisma*. But, since neither author worked with bacteria-free cultures, the results obtained might have been partly influenced by the bacteria present. Issekutz and Issekutz (1944) did not find any increase of the respiration of *Paramecium* in the presence of glucose.

b) Addition of peptone raises the oxygen consumption by 12–15 per cent.

TABLE 6

RESPIRATION IN SALT SOLUTION CONTAINING 1 PER CENT PEPTONE AS COMPARED WITH SALT SOLUTION CONTAINING 1 PER CENT PEPTONE AND 1 PER CENT GLUCOSE

Experiment No.	Oxygen Consumption (Mm.³)		Difference (Per Cent)
	Salt Solution with 1 Per Cent Peptone and 1 Per Cent Glucose	Salt Solution with 1 Per Cent Peptone	
1	71.4	72.9	+2.2
2	98.2	96.0	−2.2
3	146.8	142.5	−2.9
4	111.0	118.5	+6.7
5	172.3	175.1	+1.6
6	204.3	207.7	+1.6
7	70.2	69.3	−1.2
8	75.3	79.3	+5.3
Mean			+1.3 $\sigma = \pm 3.2$

These values agree quite well with those given by Leichsenring (1925) for *P. caudatum* and Emerson (1929) for *Blepharisma*.

These authors found that after the addition of peptone the respiration increased by 13 per cent for *P. caudatum* and by over 10–20 per cent for *Blepharisma*. These figures are much lower than those given for *T. geleii* by Ormsbee (1942), who found that, when peptone was added, the oxygen consumption increased 210 per cent in the exponential

phase of the culture and 370 per cent in the stationary phase. Similarly, Pace and Lyman (1947) in *T. geleii* obtained an increase of oxygen consumption by over 100 per cent on adding peptone to the medium. But, since the values found by Ormsbee and by Pace and Lyman are so much higher than those reported by other authors for the same species, one might presume that the peptone used by them contained some contamination which caused an increase of the respiration.

TABLE 7

OXYGEN CONSUMPTION OF AMOEBAE STARVED FOR 24 HOURS

Experiment No.	O_2 mm^3/Hour/10^7 Amoebae		Increase of Respiration in Peptone and Glucose Solutions (Per Cent)
	Salt Solution Alone	Salt Solution with 1 Per Cent Peptone and 1 Per Cent Glucose	
1.....	87.5	95.7	+ 9.4
2.....	96.4	116.4	+20.7
3.....	87.0	104.5	+19.7
4.....	63.9	77.2	+20.9
5.....	81.7	101.1	+23.6
6.....	85.9	109.2	+27.0
7.....	106.3	130.5	+22.7
8.....	73.0	94.9	+29.9
9.....	65.0	82.1	+26.3
10.....	91.6	109.7	+19.7
Mean..	83.8 $\sigma = \pm$ 13.4	102.1 $\sigma = \pm$ 15.7	+22.0 $\sigma = \pm$ 5.6

In this connection the observation of Issekutz and Issekutz (1944) should be mentioned. They found that the addition of dead yeast cells raises the respiration not only on account of their nutritive value but also on account of their vitamin content. Thus it is possible that the increase of the oxygen consumption observed by Ormsbee was caused by vitamins present in the peptone preparation used by him.

INFLUENCE OF STARVATION ON RESPIRATION

In order to determine the influence of starvation on the respiration, the centrifuged amoebae were washed in the salt solution and suspended in 25 ml. of fresh salt solution. Five ml. of this suspension were distributed into each of five 50-ml. Erlenmeyer flasks and were starved for 24 hours at 26° C.

This arrangement was chosen in order to avoid a high liquid column in the flasks and the consequent lack of oxygen. The next day, the amoebae were again centrifuged, washed, suspended in fresh salt solution, and, in the usual way, distributed among the various manometers. Table 7 shows the results of comparing the respiration of these amoebae in salt solution with and without peptone and glucose. Each figure represents the average of two manometers. It can be seen from this table that the average oxygen consumption of starved amoebae in an inorganic salt solution amounts to 83.8 mm^3 O_2/hour/10^7 amoebae ($\sigma = \pm 13.4$); it is, therefore, 18.0 per cent lower than that of unstarved amoebae in an identical solution.

In solutions containing peptone and glucose the average oxygen consumption of starving amoebae is 102.1 mm^3 O_2/hour/10^7 amoebae ($\sigma = \pm 15.7$); it is, therefore, 12.9 per cent lower than those of unstarved amoebae in an identical solution. Hence the difference between the respiration in salt solution and that in solutions containing peptone and glucose amounts to +22.0 per cent for starving amoebae and to +15 per cent for unstarved amoebae. It can be seen that, under the experimental conditions described, the amoebae were enabled to compensate partly for the deficit caused by starvation by an increased food intake (apparently peptone solution) after 1 or

1½ hours. That in this case, too, glucose was not immediately used may be seen from the results of an experiment in which the respiration of starved amoebae in salt solution was compared with respiration in the presence of glucose. The difference, in this case, amounted to +0.5 per cent, a figure which has no practical significance.

This difference between the amoebae which were starved for 24 hours and those which were kept at standard conditions agrees with the figures found by Lund (1918*a*, *b*) and Leichsenring (1925) for *Paramecium*. It is, however, considerably smaller than that found by Pace and Belda (1944) for *P. carolinensis*. In that case the difference in the respiration between the well-fed and the starved amoebae was found to be nearly 200 per cent.

INFLUENCE OF THE pH ON THE RESPIRATION

In all experiments on the influence of pH on respiration a 0.15 per cent NaCl–0.005 M phosphate buffer solution either with or without peptone and glucose was used, and the experiments were carried out at pH 6.0 and pH 8.0, respectively. These pH concentrations were chosen because earlier experiments (Reich, 1936) had shown that the growth optimum is approximately at pH 6.0 while at pH 8.0 the cultures showed only a weak development.

It was hoped, therefore, that, if any differences in respiration were caused by different pH, it would be easiest to ascertain them in these particular two concentrations of hydrogen ions.

First, the respiration in the salt solution without organic additions was compared at pH 6.0 and pH 8.0. The results, summed up in Table 8, show that the respiration at pH 8.0 was, on the average, 1.04 per cent ($\sigma = \pm 2.1$) higher than at pH 6.0. As the standard deviation is so high, this difference possesses hardly any significance. However, when a similar experiment was made in a solution containing peptone and glucose, the difference between the respiration at pH 6.0 and at pH 8.0 amounted to −4.15 per cent ($\sigma = \pm 3.94$) (see Table 9).

TABLE 8

RESPIRATION OF AMOEBAE IN 0.15 PER CENT NaCl AND 0.005 M PHOSPHATE BUFFER AT pH 8.0 AS COMPARED WITH RESPIRATION AT pH 6.0

Experiment No.	Oxygen Consumed (Mm.³)		Difference at pH 8.0 (Per Cent)
	pH 6.0	pH 8.0	
1	141.8	146.5	+3.3
2*a*	126.1	131.9	+4.6
2*b*	128.9	132.6	+2.8
3*a*	158.0	162.1	+2.6
3*b*	170.5	173.9	+1.9
4*a*	98.1	97.7	−0.4
4*b*	102.5	101.0	−1.4
5*a*	81.2	79.6	−1.9
5*b*	116.4	112.5	−3.0
6*a*	111.1	116.7	+5.0
6*b*	110.7	115.9	+4.6
7*a*	74.3	72.1	−2.9
7*b*	74.6	74.6	0.0
8*a*	70.9	70.9	0.0
8*b*	71.6	72.2	+0.8
9*a*	125.2	125.2	0.0
9*b*	124.2	125.2	+0.7
10*a*	137.4	140.1	+1.9
10*b*	143.0	143.4	+0.2
11*a*	149.9	152.1	+1.4
11*b*	153.6	155.3	+1.1
12	124.6	127.5	+2.3
13*a*	103.6	105.6	+1.9
13*b*	98.2	99.3	+1.1
14	72.3	73.6	+1.8
15	110.3	108.5	−1.6
Mean			+1.04 $\sigma = \pm 2.1$

If the results obtained in these two solutions are compared, one comes to the conclusion that at pH 8.0 it is not the respiration itself which was affected but that the supply of fresh nutritive material was hindered either by disturbing its intake by the amoebae or, what seems even more likely, by unfavorably influ-

encing the action of the proteolytic ferments of the cell. In order to test this assumption, the experiments on the difference of the respiration in salt solutions with and without peptone and glucose were repeated, but this time in two parallel series, one at pH 6.0 and the other at pH 8.0 (Tables 10 and 11). A comparison of the results obtained proved that, at pH 6.0, an addition of peptone-glucose increases the respiration by 15.9 per cent ($\sigma = \pm 2.0$), while at pH 8.0 the increase amounts to only 13.1 per cent ($\sigma = \pm 2.3$). This difference is still greater if, instead of the mean difference calculated for the entire duration of the experiment (4 hours), the differences calculated separately for each consecutive hour of the experiment are considered (see Table 12). It can be seen that for the first hour of the experiment the average difference of the respiration in the solution containing glucose-peptone amounted to +20.2 per cent ($\sigma = \pm 3.5$) at pH 6.0, while it was only +16.2 per cent ($\sigma = \pm 3.7$) at pH 8.0. There is again the same difference of about 4 per cent between the respiration at pH 6.0 and at pH 8.0 that was found in the experiments summarized in Table 9. But it can also be seen that in the course of the experiments the decrease in the respiration is greater at pH 6.0 than at pH 8.0. This might be caused by the accumulation, in the solution, of harmful substances produced by the metabolism of the amoebae.

TABLE 9

RESPIRATION OF AMOEBAE IN 0.15 PER CENT NaCl, 1 PER CENT PEPTONE, 1 PER CENT GLUCOSE, AND 0.005 M PHOSPHATE BUFFER AT pH 8.0, AS COMPARED WITH RESPIRATION AT pH 6.0

Experiment No.	Oxygen Consumed (Mm.³)		Difference at pH 8.0 (Per Cent)
	pH 6.0	pH 8.0	
1	127.8	121.4	− 5.0
2	117.3	102.1	− 4.4
3	67.2	66.8	− 0.5
4*a*	113.4	108.9	− 3.9
4*b*	103.9	103.2	− 0.6
5*a*	105.0	105.5	+ 0.4
5*b*	101.9	102.0	0.0
6*a*	126.0	119.2	− 5.4
6*b*	122.4	120.5	− 1.5
7*a*	138.1	124.3	− 9.9
7*b*	122.5	117.4	− 4.1
7*c*	119.5	118.1	− 1.1
8*a*	171.1	163.7	− 4.3
8*b*	153.8	148.9	− 6.1
9*a*	167.1	159.6	− 4.4
9*b*	157.7	151.1	− 4.1
10*a*	111.0	107.5	− 3.1
10*b*	113.7	111.5	− 1.0
10*c*	99.2	96.7	− 2.5
11*a*	156.9	140.4	−10.5
11*b*	140.3	126.9	− 9.5
11*c*	139.3	130.2	− 6.5
12*a*	160.2	142.4	−11.1
12*b*	155.0	137.0	−11.6
12*c*	149.1	131.5	−11.1
13*a*	108.1	101.2	− 6.3
13*b*	98.4	97.3	− 1.1
13*c*	95.9	95.2	− 0.7
13*g*	90.3	89.1	− 1.3
14*a*	145.7	146.5	+ 0.5
14*b*	139.6	143.8	+ 3.0
Mean			− 4.15 $\sigma = \pm$ 3.94

In the literature we find only a few accounts of the influence of pH on the respiration of Protozoa, and even those few accounts are contradictory. Hall (1941) found that, in peptone-yeast extract, *Colpidium campylum* reaches its maximum of respiration at pH 5.5, and this decreases gradually with the rising or falling pH. His results were somewhat different according to the buffer used; it is possible, therefore, that they were partly influenced by the buffer solution.

Issekutz and Issekutz (1944) found, on the other hand, that *Paramecium* is not very sensitive to changes of the pH. The results described in this paper for *M. palestinensis* show that in this respect its behavior is similar to that of *Paramecium*.

THE RESPIRATORY QUOTIENT

It is due to the technical difficulties connected with the determination of the

R.Q. of isolated cells that the results published on the respiration of Protozoa are very contradictory. Thus, Hutchens (1941) found for *Chilomonas paramecium* a R.Q. of 0.77–0.93, while Mast, Pace, and Mast (1936) had reported for the same species a R.Q. of 0.283–0.370. For *T. geleii*, Pace and Lyman (1947) found fluctuations of the R.Q. between 0.95 and 2.81. Probably the main reason for these contradictory figures lies in shortcomings of the technique; in particular, the retention of carbon dioxide by the solution used, which varies in each case, appears to have been left out of account quite frequently. In order to avoid this source of error, the method recommended by Warburg was used in the experiments described below. For each determination three manometers were used (besides the thermobarometer). In all respiratory vessels 0.2 ml. 3M H_2SO_4 was introduced into the side bulb. The first of the three manometers, which contained 0.2 ml.

TABLE 10

RESPIRATION OF AMOEBAE IN SOLUTION OF 0.15 PER CENT NaCl, 1 PER CENT PEPTONE, 1 PER CENT GLUCOSE, AND 0.005 M PHOSPHATE BUFFER OF pH 6.0 AS COMPARED WITH RESPIRATION IN SIMILAR SOLUTION BUT WITHOUT PEPTONE AND GLUCOSE

Experiment No.	Consecutive Hours of Experiment	Oxygen Consumption (Mm.³)		Difference per Single Hour (Per Cent)	Mean Difference (Per Cent)
		Salt Solution Alone	Salt Solution with 1 Per Cent Peptone and 1 Per Cent Glucose		
1	1	148.5	172.0	+15.8	+13.2
	2	135.9	155.3	+14.2	
	3	131.5	145.8	+10.9	
	4	125.9	140.6	+11.7	
2	1	137.1	165.6	+20.0	+15.8
	2	132.6	153.9	+14.6	
	3	128.6	142.8	+11.0	
	4	127.5	146.8	+15.1	
3	1	115.1	139.1	+21.5	+15.7
	2	108.7	124.6	+14.6	
	3	106.2	120.6	+13.5	
	4	101.8	115.1	+13.3	
4	1	151.7	177.5	+16.9	+14.5
	2	144.0	165.8	+15.1	
	3	138.0	154.8	+12.1	
	4	132.2	150.2	+13.6	
5	1	149.0	169.0	+21.4	+17.7
	2	131.8	155.3	+17.7	
	3	125.1	144.2	+15.2	
	4	123.4	143.0	+15.8	
6	1	103.1	129.3	+25.5	+18.7
	2	97.7	115.7	+18.9	
	3	100.8	116.9	+15.9	
	4	95.7	109.8	+14.7	
Mean					+15.9 $\sigma = \pm$ 2.0

KOH in the central compartment, served in the usual way for the determination of the oxygen consumption. In the second manometer, containing distilled water instead of KOH, the free carbon dioxide was first measured; at the close of the experiment, by careful tilting of the manometer, the sulphuric acid was poured from the side bulb into the main vessel, and in this way the carbon dioxide retained by the solution was set free. In the third manometer, containing distilled H_2O like the second, the acid was poured from the side bulb into the main vessel right at the beginning of the experiment. Thus, manometer 3 served to measure the carbon dioxide present in the solution at the beginning of the experiment. The volume of carbon dioxide present at the beginning of the experiment, as measured in manometer 3, was subtracted from the total present at the end of the experiment, as measured in manometer 2. The difference represents the carbon dioxide

TABLE 11

RESPIRATION OF AMOEBAE IN A SOLUTION OF 0.15 PER CENT NaCl, 1 PER CENT PEPTONE, 1 PER CENT GLUCOSE, AND 0.005 M PHOSPHATE BUFFER AT pH 8.0 AS COMPARED WITH RESPIRATION IN SIMILAR SOLUTION BUT WITHOUT PEPTONE AND GLUCOSE

Experiment No.	Consecutive Hours of Experiment	Oxygen Consumption (Mm.³)		Difference per Single Hour (Per Cent)	Mean Difference (Per Cent)
		Salt Solution Alone	Salt Solution with 1 Per Cent Peptone and 1 Per Cent Glucose		
1	1	118.4	135.0	+14.0	+10.6
	2	120.1	130.2	+ 8.3	
	3	117.0	127.9	+ 8.3	
	4	120.8	133.9	+10.8	
2	1	118.9	137.6	+13.7	+10.8
	2	123.1	134.3	+ 9.1	
	3	118.7	128.5	+ 8.2	
	4	116.3	128.5	+10.4	
3	1	120.5	151.8	+16.3	+14.2
	2	129.5	145.4	+13.8	
	3	127.5	139.2	+ 9.2	
	4	126.1	139.1	+ 8.7	
4	1	110.6	127.2	+14.5	+13.2
	2	111.0	124.7	+12.3	
	3	107.6	119.6	+11.8	
	4	103.3	118.5	+14.7	
5	1	104.6	120.7	+15.4	+13.6
	2	98.8	111.7	+12.9	
	3	97.4	108.8	+11.7	
	4	92.4	105.3	+12.6	
6	1	123.1	151.9	+23.4	+16.7
	2	125.4	147.0	+17.1	
	3	121.3	136.2	+13.1	
	4	119.0	135.7	+14.0	
Mean					+13.1 $\sigma = \pm$ 2.3

produced during the experiment, including both free carbon dioxide and carbonic acid retained in the solution. (For details of the technique cf. Dixon, 1943, p. 64.)

The NaCl and buffer solution, described above, with or without peptone and glucose was used in these experiments, too. The experiments were carried out in two parallel series, at pH 6.0 and at pH 8.0, respectively. Tables 13 and 14 show the results obtained in solutions free of organic additions at pH 6.0 and pH 8.0, respectively. A comparison of these two tables reveals that the average R.Q. amounted to 0.832 at pH 6.0 and 0.841 at pH 8.0. The slight difference between the R.Q.'s obtained with different pH is certainly not significant.

Tables 15 and 16 show a similar series of experiments in a solution containing 1 per cent glucose. The R.Q. in this case amounted to 0.859 at both pH 6.0 and pH 8.0.

Tables 17 and 18 contain the results of experiments carried out in solutions containing 1 per cent peptone and 1 per cent glucose, and they show a R.Q. of 0.864 at pH 6.0 and of 0.786 at pH 8.0, while Tables 19 and 20 show the results of experiments in a solution containing 1 per cent peptone and no sugar. These latter experiments yielded a R.Q. of 0.871 at pH 6.0 and one of 0.823 at pH 8.0. Even if the differences between the R.Q.'s at different pH in the two last-mentioned series of experiments are statistically significant, I doubt whether they indicate real differences in the metabolism. It might rather be assumed that, in solutions containing peptone and buffered by a phosphate buffer of pH 8.0, not all the carbon dioxide formed was set free by the addition of sulphuric acid and that the differences, though statistically significant, were caused only by the technique employed.

TABLE 12

COMPARISON OF THE RESULTS OF THE EXPERIMENTS SUMMED UP IN TABLES 10 AND 11

The figures represent the mean differences from six experiments. Full explanation is given in the text.

Consecutive Hours of Experiments	Mean Difference between Respiration in Peptone- and Glucose-containing Solution and Salt Solution Alone		Difference between Results at pH 8.0 and at pH 6.0
	pH 6.0 (Per Cent)	pH 8.0 (Per Cent)	
1	$+20.2\ (\sigma = \pm 3.5)$	$+16.2\ (\sigma = \pm 3.7)$	-4.0
2	$+15.8\ (\sigma = \pm 2.0)$	$+12.2\ (\sigma = \pm 3.2)$	-3.6
3	$+13.1\ (\sigma = \pm 2.1)$	$+10.4\ (\sigma = \pm 2.1)$	-2.7
4	$+14.0\ (\sigma = \pm 1.5)$	$+11.8\ (\sigma = \pm 2.3)$	-2.2

The comparative agreement in these values of the R.Q. measured under such different conditions furnished another proof of the assumption that proteins or protein-like substances are the main, or even the only, source of energy for the metabolism of *M. palestinensis*.

The quantities of retained carbon dioxide which were found are very instructive. They show that even in a solution free of peptone, at pH 6.0, an average of 6.3 per cent of the carbon dioxide formed was retained, and in some of the experi-

TABLE 13

RESPIRATORY QUOTIENT, FREE CARBON DIOXIDE, AND RETAINED CARBON DIOXIDE IN 0.15 PER CENT NaCl, 0.005 M PHOSPHATE BUFFER SOLUTION AT pH 6.0

Experiment No.	Oxygen Consumed (Mm.³)	Free Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Per Cent)	Total Carbon Dioxide Produced (Mm.³)	R.Q.
1	256.7	200.9	13.7	5.9	214.6	0.836
2	234.0	175.0	19.6	10.1	194.6	0.831
3	183.6	145.3	8.1	5.2	153.4	0.835
4	201.1	160.0	6.5	4.0	166.5	0.825
Mean				6.3 $\sigma = \pm$ 2.6		0.832 $\sigma = \pm 0.004$

TABLE 14

RESPIRATORY QUOTIENT, FREE CARBON DIOXIDE, AND RETAINED CARBON DIOXIDE IN 0.15 PER CENT NaCl, 0.005 M PHOSPHATE BUFFER SOLUTION AT pH 8.0

Experiment No.	Oxygen Consumed (Mm.³)	Free Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Per Cent)	Total Carbon Dioxide Produced (Mm.³)	R.Q.
1	324.7	224.4	45.4	16.8	269.8	0.831
2	334.2	235.7	54.8	18.1	290.5	0.866
3	205.5	132.6	39.7	23.0	172.3	0.838
4	239.9	159.6	39.1	19.6	198.7	0.828
Mean				19.4 $\sigma = \pm$ 2.7		0.841 $\sigma = \pm 0.017$

TABLE 15

RESPIRATORY QUOTIENT, FREE CARBON DIOXIDE, AND RETAINED CARBON DIOXIDE IN 0.15 PER CENT NaCl, 0.005 M PHOSPHATE BUFFER, AND 1 PER CENT GLUCOSE SOLUTION AT pH 6.0

Experiment No.	Oxygen Consumed (Mm.³)	Free Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Per Cent)	Total Carbon Dioxide Produced (Mm.³)	R.Q.
1	258.2	207.9	18.2	8.0	226.1	0.875
2	219.4	172.2	19.8	10.3	192.0	0.875
3	291.1	222.8	21.4	8.7	244.2	0.838
4	277.2	214.6	20.7	9.6	235.3	0.848
Mean				9.1 $\sigma = \pm$ 1.0		0.859 $\sigma = \pm 0.020$

TABLE 16

RESPIRATORY QUOTIENT, FREE CARBON DIOXIDE, AND RETAINED CARBON DIOXIDE IN 0.15 PER CENT NaCl, 0.005 M PHOSPHATE BUFFER, AND 1 PER CENT GLUCOSE SOLUTION AT pH 8.0

Experiment No.	Oxygen Consumed (Mm.³)	Free Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Per Cent)	Total Carbon Dioxide Produced (Mm.³)	R.Q.
1	208.4	144.4	34.8	19.4	179.2	0.860
2	212.1	142.2	42.1	22.8	184.3	0.868
3	307.2	224.8	36.4	13.9	261.2	0.850
4	340.1	244.5	47.6	16.2	292.1	0.858
Mean				18.0 $\sigma = \pm$ 3.9		0.859 $\sigma = \pm 0.007$

TABLE 17

RESPIRATORY QUOTIENT, FREE CARBON DIOXIDE, AND RETAINED CARBON DIOXIDE IN 0.15 PER CENT NaCl, 0.005 M PHOSPHATE BUFFER, 1 PER CENT PEPTONE, AND 1 PER CENT GLUCOSE SOLUTION AT pH 6.0

Experiment No.	Oxygen Consumed (Mm.³)	Free Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Per Cent)	Total Carbon Dioxide Produced (Mm.³)	R.Q.
1	226.0	167.7	28.5	14.5	196.2	0.868
2	253.8	209.2	10.3	4.6	219.5	0.864
3	163.1	115.8	20.5	15.2	136.3	0.835
4	201.1	156.1	24.4	13.5	180.5	0.897
5	187.2	143.9	16.7	10.4	160.6	0.857
Mean				11.6 $\sigma = \pm$ 4.3		0.864 $\sigma = \pm 0.022$

TABLE 18

RESPIRATORY QUOTIENT, FREE CARBON DIOXIDE, AND RETAINED CARBON DIOXIDE IN 0.15 PER CENT NaCl, 0.005 M PHOSPHATE BUFFER, 1 PER CENT PEPTONE, AND 1 PER CENT GLUCOSE SOLUTION AT pH 8.0

Experiment No.	Oxygen Consumed (Mm.³)	Free Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Per Cent)	Total Carbon Dioxide Produced (Mm.³)	R.Q.
1	200.4	111.3	46.9	42.1	158.2	0.789
2	220.9	86.6	103.8	54.5	190.4	0.861
3	180.6	81.6	68.0	45.4	149.6	0.828
4	89.2	22.0	41.2	65.1	63.2	0.708
5	212.8	75.4	86.7	53.4	162.1	0.761
Mean				52.1 $\sigma = \pm$ 9.0		0.786 $\sigma = \pm 0.059$

TABLE 19

RESPIRATORY QUOTIENT, FREE CARBON DIOXIDE, AND RETAINED CARBON DIOXIDE IN 0.15 PER CENT NaCl, 0.005 M PHOSPHATE BUFFER, AND 1 PER CENT PEPTONE SOLUTION AT pH 6.0

Experiment No.	Oxygen Consumed (Mm.³)	Free Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Per Cent)	Total Carbon Dioxide Produced (Mm.³)	R.Q.
1	288.2	231.4	55.7	13.7	267.1	0.926
2	359.1	279.4	33.4	10.6	312.8	0.871
3	296.2	223.7	27.8	11.0	251.8	0.850
4	273.5	217.6	42.7	16.4	260.3	0.951
5	269.1	202.6	27.7	12.0	230.3	0.855
6	237.0	193.2	13.5	6.5	206.7	0.872
7	223.8	177.0	15.0	7.8	192.0	0.857
8	226.0	186.6	5.2	2.7	191.8	0.848
9	231.1	189.1	8.8	4.4	197.9	0.856
10	230.4	193.8	6.3	3.1	200.1	0.868
11	209.9	129.5	45.3	25.9	174.8	0.832
12	217.2	152.5	62.5	28.6	215.0	0.989
13	175.5	120.8	26.4	17.9	147.2	0.838
14	212.1	145.4	30.1	17.1	175.5	0.827
15	197.5	144.2	20.6	12.4	164.9	0.834
Mean				12.6 $\sigma = \pm$ 7.6		0.871 $\sigma = \pm 0.035$

TABLE 20

RESPIRATORY QUOTIENT, FREE CARBON DIOXIDE, AND RETAINED CARBON DIOXIDE IN 0.15 PER CENT NaCl, 0.005 M PHOSPHATE BUFFER, AND 1 PER CENT PEPTONE SOLUTION AT pH 8.0

Experiment No.	Oxygen Consumed (Mm.³)	Free Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Mm.³)	Retained Carbon Dioxide (Per Cent)	Total Carbon Dioxide Produced (Mm.³)	R.Q.
1	330.6	217.5	58.2	21.1	275.7	0.833
2	346.0	213.3	73.7	25.6	287.0	0.829
3	242.1	121.8	63.4	22.2	185.2	0.764
4	247.2	146.0	57.8	28.3	203.8	0.824
5	231.8	130.8	56.4	30.1	187.2	0.807
6	250.9	144.0	78.0	35.1	222.0	0.884
7	193.1	128.4	60.4	31.9	188.8	0.977
8	280.0	173.8	66.6	27.7	240.4	0.858
9	207.7	96.6	67.9	41.4	164.5	0.792
10	208.4	102.8	63.5	38.1	166.3	0.798
11	184.3	42.5	95.0	69.0	137.5	0.746
12	220.8	85.0	105.9	52.6	190.9	0.864
13	164.5	56.8	68.0	54.4	124.9	0.753
14	207.7	102.8	63.7	38.2	166.5	0.801
15	218.7	105.4	75.7	41.7	181.1	0.828
Mean				35.8 $\sigma = \pm 13.3$		0.823 $\sigma = \pm 0.058$

ments the volume retained amounted to 10.1 per cent. In solutions containing peptone the amount of carbon dioxide retained was much greater, and, at pH 8.0, it rose in some cases to 69.0 per cent of the total carbon dioxide formed by respiration. These results show that care must be exercised in interpreting manometric determinations of carbon dioxide formed by the respiration of single cells. For this reason there is no need to discuss more fully the figures given by former investigators and reviewed by Jahn (1941). I only want to call attention to the more recent results obtained in *Paramecium* by Pace and Kimura (1944), who maintain that the R.Q. is dependent on temperature. In *P. caudatum* the R.Q. at 15° C. was 0.70, rising to 0.99 at 35° C.; for *P. aurelia* the figures were 0.73 at 15° C. and 0.90 at 30° C. If further investigations should confirm these accounts, they would provide us with an explanation of much contradictory evidence contained in the papers published. On the other hand, Pace and Belda (1944) in *Pelomyxa carolinensis* found a R.Q. of 0.87 for well-fed amoebae and 0.56 for starved amoebae. The considerable fluctuations of the R.Q. observed by Pace and Lyman (1947) in *T. geleii* have already been mentioned.

INFLUENCE OF OXYGEN TENSION ON RESPIRATION

The experiments on the influence of oxygen tension on the respiration of *M. palestinensis* were carried out with various oxygen-nitrogen mixtures. For this purpose the desired gas mixture was prepared in a gasometer, and, with the help of an apparatus similar to the one described by Dixon and Tunnicliffe (1923), the manometers were filled with this gas mixture. Although these authors recommended the use of their apparatus only for differential manometers, it was possible to adapt it for use with the Warburg manometer. There is one essential precaution: before the tap which is connected with the arm of the manometer carrying the respiratory vessel is closed, the surplus of gas must be removed with the aid of the two-way tap. Otherwise the fluid in the manometer will be pressed through the open arm when the tap is closed. After the manometer was filled, part of the gas mixture was conveyed into a gas burette, where the exact oxygen content in the gas mixture was determined. In order to permit an equilibration of the temperature, the first readings of the manometers were taken 15 minutes after the manometers had been filled with the gas mixtures and inserted into the water bath. Every half-hour they were refilled with the gas mixture without being removed from the water bath, and in this way each experiment was carried out simultaneously in two manometers involving three to four half-hourly readings. All experiments were carried out in the salt-buffer solutions and in two series, at pH 6.0 and at pH 8.0, respectively. The respiration in two manometers filled with air served as a control; the results, given in Tables 21 and 22, are expressed as percentages of the respiration in the control experiments carried out simultaneously.

A study of these tables reveals, first of all, that, even at a low oxygen tension, the respiration is not influenced by the pH. Furthermore, we see that the respiration at oxygen tensions between 50 and 5 per cent equals the respiration in air and that the influence of the oxygen tension becomes noticeable only at oxygen concentrations below 5 per cent. In gas mixtures containing 2–3 per cent oxygen the respiration of the amoebae still amounted to approximately 50 per cent of the normal respiration in air.

The results with gas mixtures contain-

ing less than 2 per cent oxygen became more and more uncertain, probably because at these concentrations the amoebae were rapidly using up the oxygen present in the respiratory vessel and, consequently, the oxygen content of the gas mixture in the respiratory vessel changed very quickly. The lowest figure of the respiration was measured during an experiment at 1.5 per cent oxygen tension, where it still amounted to 23.5 per cent of the normal respiration. Yet this figure seems far too low, since in the subsequent experiment carried out at 1.1 per cent there still remained 42.2 per cent of the normal respiration.

TABLE 21

RESPIRATION OF AMOEBAE AT VARIOUS OXYGEN TENSIONS

In a 0.15 per cent NaCl and 0.005 M phosphate buffer solution of pH 6.0; each figure represents an average of the readings of two manometers.

Oxygen Tension (Per Cent)	Respiration in Percentage of Normal
50.0	100.0
9.0	100.0
5.7	100.0
4.5	91.9
4.4	93.3
4.1	89.2
4.0	86.3
3.8	85.3
3.3	87.3
2.8	52.9
2.7	67.2
2.7	56.5
2.6	57.8
2.3	59.0
2.3	69.2
2.3	52.3
2.2	69.0
1.8	44.9
1.7	54.5
1.5	24.5
1.1	42.2

The influence of the oxygen tension on the respiration of Protozoa has been investigated in a few cases only. Lund (1918*a*, *b*) and Amberson (1928) found independence of the oxygen tension for *Paramecium;* and similar results were obtained by Adolph (1929) for *Colpoda*, which under a pressure of 5 mm. HgO_2 (about 0.7 per cent O_2) still showed 31 per cent of the normal respiration. Specht (1934) found for *Spirostomum*, at 100 per cent O_2, 151 per cent of the respiration in air and, at 0.5 per cent O_2, still 41 per cent of the respiration in air.

In connection with these results some attempts have been made to calculate the amount of oxygen actually at the disposal of the cell, from the oxygen pressure of the medium, the coefficient of diffusion, the volume of the cell, and the oxygen consumption (Adolph, 1929; Rashevsky, 1940; Jahn, 1941).

TABLE 22

RESPIRATION OF AMOEBAE AT VARIOUS OXYGEN TENSIONS

In a 0.15 per cent NaCl and 0.005 M phosphate buffer solution of pH 8.0; each figure represents an average of the readings of two manometers.

Oxygen Tension (Per Cent)	Respiration in Percentage of Normal
50.0	100.0
8.5	100.0
5.5	97.5
5.2	100.0
4.3	81.5
4.2	85.2
4.0	82.0
4.0	92.6
3.7	72.6
3.6	76.2
3.5	78.7
3.0	55.7
2.9	63.0
2.6	55.2
2.6	37.5
2.5	38.3
2.3	67.6
2.1	55.8
2.0	66.3
1.7	43.0

These calculations are based on the assumption that the cell has a regular shape and, furthermore, that oxygen is transported inside the cells mainly by means of diffusion. For certain Protozoa, viz.,

Paramecium, the first of these suppositions may be correct, although the second is certainly untenable, since the plasma currents undoubtedly play an important part in the transport of oxygen inside the cell. Neither of these assumptions may be applied to a flattened amoeboid cell of irregular shape exhibiting strong plasmatic currents. It is therefore all the more remarkable that *M. palestinensis*, in spite of its pronounced plasma currents, shows a much larger decrease of the respiration at lower oxygen tensions than was measured for ciliates.

DISCUSSION

It has already been stressed that, when dealing with the experimental results, a comprehensive survey of the various investigations on protozoan respiration is not possible at the present stage, in view of the numerous contradictory conclusions. Many of these contradictions are doubtless due to the employment of imperfect techniques. In older investigations a possible modification of the results by bacterial contamination can never be excluded. It must also be borne in mind that protozoan cells of different species, just like the different metazoan cells, do not allow a real comparison of the results which can be reduced to a common denominator. Moreover, we have at present very little exact information regarding the physiological differences distinguishing different strains of the same protozoan species. However, the investigations of Kidder and Dewey (1945) on the physiological differences between various strains of *T. geleii* have demonstrated clearly that much of the contradiction in the experimental results obtained with the same species is due to the employment of different strains by different authors. There is no need to point out that in Protozoa, and particularly in species that rarely undergo sexual processes, any mutation offering an advantage under special environmental conditions runs a good chance of survival; hence it is to be expected that strains originating from different localities may exhibit considerable physiological differences, which are not necessarily accompanied by morphological differences.

Under these circumstances it is not remarkable that there are discrepancies between the results of various workers; on the contrary, it appears rather noteworthy that these discrepancies are relatively small. Most surprising is the fact that the oxygen consumption per individual of *M. palestinensis* is so closely similar to that of *P. carolinensis*, viz., 117.3 mm^3 O_2/hour/10^7 amoebae at 27° C. for the former and 132.4 mm^3 O_2/hour/10^7 amoebae at 30° C. for the latter (Pace and Belda, 1944). Since the volume of a *Pelomyxa* cell is many times larger than that of a *Mayorella* cell, *Pelomyxa* should be expected to have a correspondingly larger oxygen consumption. In this case, too, only a comparison of the respiration of as many different species of amoeba as possible can show whether we are dealing with a coincidence or with some general rule.

The present experiments on the influence of the substrate on the respiratory rate, as well as the respiratory quotient, which remains relatively constant under vastly different external conditions, indicate that protein-like substances constitute the main food of *M. palestinensis*. While in the present investigations the R.Q. of the amoebae as well as of the cysts remained constant under different experimental conditions, most previous authors report great fluctuations in the R.Q. Here, again, it appears doubtful whether the carbon dioxide registered corresponds to the amount produced in

respiration, for the measuring technique was inadequate in most cases.

The difference between the oxygen consumption in purely inorganic salt solutions and that in peptone solutions, particularly for amoebae which had been starved for 24 hours, gives a fair insight into the basic metabolism and the rate of food absorption in the amoeba. We may assume that in starved amoebae about 20 per cent of the respired material is supplied by direct food absorption. These values closely approach those reported by earlier authors for other Protozoa. Only in two cases (Ormsbee, 1942; Pace and Lyman, 1947) was there found for *T. geleii* a much larger difference between the respiration in salt solution and that in peptone solution. Since Ormsbee worked with strain "H" and Pace and Lyman with strain "W" (cf. Kidder and Dewey, 1945), it would appear that specific differences between strains are not involved, especially since Hall (1941), experimenting with the same strain "H," found much lower values. Apparently, in this case the difference between the peptone preparations used plays a decisive role. Clear-cut results will be obtained only when the commercial peptone preparations are replaced by chemically well-defined substances and when experimental techniques are standardized.

It was previously observed (Reich, 1935, 1936) that cultures of *M. palestinensis* reach a much higher maximum population density on addition of glucose than they do in sugar-free media, though no acid is formed in appreciable quantities. The present investigations on the respiration of *M. palestinensis* show that the addition of glucose to the nutritive solution affects neither the oxygen consumption nor the respiratory quotient. It appears fairly certain that glucose is not directly involved in the respiratory metabolism of *M. palestinensis*. This gives rise to the question of how glucose may affect the nutritive medium so that it will support the development of a much higher population density.

It may be pointed out once more that Issekutz and Issekutz (1944), in contrast to the previous results of Leichsenring (1925), did not report influence of glucose addition on the respiration of *Paramecium*, while Kidder and Dewey (1945) found that all their strains of *T. geleii* were able to ferment glucose.

Previous experiments (Reich, 1936) showed that the maximum population density of *M. palestinensis* was about ten times higher at pH 6.0 than at pH 8.2. The present results indicate that in peptone-free solutions there is no difference between the respiration at pH 6.0 and at pH 8.0; in solutions containing peptone there was clearly a lower oxygen consumption at pH 8.0. The conclusion seems warranted that a lower pH interferes with the absorption or the digestion of food, without affecting respiration as such.

In previous culture experiments (Reich, 1936) it was shown that in semi-solid nutritive media the zone of growth is clearly dependent on pH. In a lower pH the growth zone is situated deeper in the test tube than at a higher pH. Hence it was assumed that the optimum oxygen pressure for *M. palestinensis* varies with the pH and that the amoebae actively migrate into the zone of optimum oxygen pressure. The present experiments were carried out at different oxygen concentrations as well as at both pH 6.0 and pH 8.0, and they indicated that even at relatively low oxygen pressures (down to 1.5 per cent of the total gas volume) there was no direct relation between respiratory rate and pH. However, it should be borne in mind that, even at the lowest oxygen pressures employed, the actual concentration of oxygen in the solution is

much higher in the constantly shaken manometers than in test-tube cultures, where the amoebae are covered by a column of immobile liquid 2–3 cm. in height. Under these circumstances conditions are almost completely anaerobic, for the diffusion rate of oxygen is too low to replace its consumption by the amoebae.

It will be the object of future experiments to investigate the metabolism of *M. palestinensis* under anaerobic or almost anaerobic conditions.

SUMMARY

The respiration of an amoeba, *M. palestinensis*, in bacteria-free culture was investigated under various conditions, and the following results were obtained:

1. In nutrient solution containing 1 per cent peptone and 1 per cent glucose the respiration amounted to 117.3 mm^3 O_2/hour/10^7 amoebae, or 101.1 mm^3 O_2/hour/10 mg.

2. The respiration of cysts in the same solution amounted to 22 mm^3 O_2/hour/10^7 cysts.

3. In salt solutions devoid of peptone and glucose the mean oxygen consumption was 101.6 mm^3 O_2/hour/10^7 amoebae.

4. After the addition of 1 per cent peptone and 1 per cent glucose the mean increase of respiration was 15.2 per cent, while after the addition of peptone alone it amounted to 11.8 per cent.

5. The addition of glucose alone had no influence on the oxygen consumption.

6. Amoebae starved for 24 hours consumed 102.1 mm^3 O_2/hour/10^7 amoebae in the solution containing peptone and glucose, and 83.8 mm^3 O_2/hour/10^7 amoebae in the peptone-free solution; thus the mean increase in the presence of peptone amounted to 22 per cent. No increase of respiration of starved amoebae in the presence of glucose could be noted.

7. In peptone- and glucose-free solutions the respiration of the amoebae increased at pH 8.0 by 1.04 per cent compared with that at pH 6.0.

8. In solutions containing peptone and glucose the respiration of the amoebae decreased by 4.5 per cent at pH 8.0 as compared with that at pH 6.0.

9. Accordingly, the difference between the respiration in peptone and glucose solutions and that in salt solutions alone was higher at pH 6.0 than at pH 8.0.

10. The mean R.Q. was 0.864. Only slight differences in the R.Q. were found under the different experimental conditions. It was demonstrated that considerable and varying amounts of carbon dioxide are retained by the solutions used.

11. Changes of oxygen tension between 50 and 5 per cent had no influence on the respiration of *M. palestinensis*. Below a 5 per cent oxygen content there was a decrease of the respiration, amounting to approximately 50 per cent of the normal respiration at 2–3 per cent oxygen content, and it dropped to nearly 30 per cent of the normal at 1 per cent oxygen.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

ENDOCRINES AND CONSTITUTION IN DOVES AND PIGEONS[1] BY OSCAR RIDDLE

Reviewed by ALBERT WOLFSON
Northwestern University

NUMEROUS studies of individual differences in avian species have dealt at length with purely morphological features and their range of variation. Relatively few studies, however, have been concerned with differences in physiological traits and the nature of these differences. The present volume, encompassing the results of twenty-four years of study, is a monumental contribution to this practically unknown field. In a broad sense the studies presented deal with the genetic nature of structural and physiological differences in races (or populations) of doves and pigeons and, as such, contribute important data, conclusions, and implications for the student of speciation and evolution.

At the outset the objective was to learn whether certain individual differences of doves and pigeons could be established as racial characteristics and, if so, to learn which of these were associated with hormonal differences. It was felt that the results of such studies would yield data with definite and valuable implications for the recognition, measurement, and evaluation of human constitutional differences.

Doves and pigeons were selected for study for a number of reasons. Predominant among these was "the fact that the degree of mongrelization present in each of these species is apparently comparable with that in the human population of an American city, and that many facts relating to constitutional factors in these birds are probably applicable to man" (p. iii). This statement is made repeatedly throughout the book.

The doves studied comprised eighteen races which were derived from two to several of fourteen original birds, and four races derived by inbreeding from four pairs of doves of different and quite distinct lineage. Classification of these races according to species represented indicates four groups: (1) mostly *Streptopelia risoria* (Blond Ring Dove) with some *Turtur orientalis* (Oriental Turtle Dove); (2) the above two types combined with *S. douraca* (Japanese Ring Dove); (3) *S. risoria* and *S. douraca;* and (4) *S. risoria* only. Most of the races were crossed with at least one other race, providing twenty-three additional hybrids for study. The pigeon material included seven well-defined, standard races, four derived groups, and sixteen hybrid types obtained from crosses between these forms.

The traits examined during the course of study included body weight, thyroid size, response to prolactin, intestinal length, pituitary size, liver size, age at sexual maturity, testis weight, egg weight, hermaphroditism, weight of heart, skeleton, and plumage, and heat production. The data on heat production are the most extensive and form Part II of the book (128 pp.). The other topics are treated in separate chapters in Part I (137 pp.). Introductory and concluding chapters make up the remainder of the book (38 pp.).

Each chapter in which data are presented begins with a review of the literature and discussion of relevant problems and concludes with a detailed section entitled "Conclusions and Summary." These latter sections are exceptionally well done and contribute greatly to the value of the book. There is no index, but it is somewhat replaced by an extensive Table of Contents; the literature is cited in footnotes. The book is well printed, well edited, and space has not been spared in the presentation of the 187 figures, 68 tables, and 6 plates.

[1] Washington, D.C.: Carnegie Institution of Washington, 1947. Pp. xi+306+187 figs. $3.00 (paper cover), $4.00 (cloth).

Weights and measurements of internal organs were taken immediately after killing by decapitation and after desanguination. The numerous and diverse data obtained (Part I) are presented effectively in tables and graphs. Facilitating ready comparison of different populations is the graphic presentation of percentile analysis of frequency distributions. Heat-production measurements were made in a modified multiple-chamber apparatus of Benedict. Great care was taken to standardize the conditions under which the tests were made. The data for each race and cross are presented lucidly and are classified according to the three temperatures used, sex, the four seasons, and age. Heat production is expressed primarily in terms of calories per kilogram per hour, but some data are given in terms of surface area. The environmental factors which were found to have the greatest importance in the physiology of doves and pigeons were feed, caging conditions, disease-producing organisms, and seasonal changes in light and temperature. That these factors were duly regarded during the course of the experiments and in the acquisition and analysis of the data attests to the validity of the measurements for purposes of comparison.

Space limitations preclude a complete review of the significant results and conclusions of these extensive studies. However, a general summary in answer to the initial objectives is in order. The results demonstrate clearly that races with different expressions of physiological traits could be segregated and that many of these traits are associated with the activities of endocrine organs. Significantly, certain expressions of traits occurred in definite combinations, and those races which possessed some of these combinations could not be maintained. Thus constitution apparently was a determining factor in survival under the prevailing conditions in the colony. It was not demonstrated, however, that the combination of traits was the cause of extinction.

With the exception of small size of testes and heart weight, extreme expressions of traits were shown to be determined genetically and not environmentally (as evidenced by the production of diverse races and the transmissibility of most of these traits in outcrosses). Few data, however, were obtained regarding the mode of inheritance. From the small original group of ring doves, races were established with the following traits: with or without a sex difference in body weight, with large or small thyroids, with long or short intestines, with high or low response to prolactin, with large or small pituitary glands, with early or late sexual maturity in females, with large or small eggs, and with high or low rate of heat production.

There can be no doubt that Dr. Riddle and his co-workers have answered admirably the questions which they posed at the outset of their investigation. In so doing, they have obtained an extensive body of significant data with important implications for several biological fields.

The author, however, goes even further in his concluding remarks regarding the human significance of these studies. The reviewer regards these human extensions as faulty and premature in the light of present biological knowledge and completely rejects them and their somewhat dangerous implications for the sociologist and politician.

No one doubts that no two men are alike. Variation is the rule in any population. What determines biological inequality, however, is not variation in constitutional factors *alone*, as the author implies, but the response of the organism to environmental factors, which the author neglects. The crux of the matter is: To what extent can these unequal traits maintain "normal" function under varying environmental conditions? The environment unquestionably influences the expression of hereditary factors not only during development but throughout the life of the organism. Adaptability of individuals to widely different conditions attests to the importance of the environment in any consideration of biological inequality; and in the case of the human species the problem is exceptionally complex because one must consider not only the physical and biotic factors in the environment but the cultural factors as well. Furthermore, one must not overlook the important problem of the degree of difference and its *biological significance*. For example, granted that there is a heritable difference in heat production, thyroid size, or length of intestine, are the relatively small differences significant from the standpoint of survival, superiority, or adaptability?

This book is a major contribution to avian biology and represents an important step forward in our definition and understanding of physiological and morphological differences in domesticated avian populations. There still remains the great task of determining the biological significance—in a broad sense—of these differences.

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